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The relationship between heat shock proteins gene expression and some antioxidants enzymes gene expression in bone marrow cells during first three months age in rats

Walaa Alaadin Mustafa

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Supervised by

Assistant Professor

Dr. Ali Saeed Hammoodi Al-chalabi

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Walaa Alaadin Mustafa

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Supervisor Acknowledgment

I certify that the preparation of this thesis entitled " **The relationship between heat shock proteins gene expression and Some antioxidants enzymes gene expression in bone marrow cells during first three months age in rats** " was carried out under my supervision at the University of Mosul / College of Veterinary Medicine, and it is part of the requirements for a master's degree in veterinary medicine / Veterinary physiology**.**

Signature:
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Linguistic acknowledgment

I certify that this letter tagged " **The relationship between heat shock proteins gene expression and Some antioxidants enzymes gene expression in bone marrow cells during first three months age in rats** " has been linguistically revised and corrected for linguistic and expressive errors. style and correctness of expression**.**

Signature:
Linguistic evaluator:
Data: / / 2022

Approval of the Head of the Physiology, Biochemistry and Pharmaceutical department

Based on the recommendations made by the supervisor and the Linguistic evaluator, I am recommending this thesis for viva.

Signature:
Name: Prof. Dr. Nashaat Galib Mustafa
Date: / /2022

Approval of the Chairman of the Graduate Studies Committee

Based on the recommendations made by the supervisor, linguistic assessor, and head of the Physiology, Biochemistry and Pharmacology branch, I nominate this thesis for viva.

Signature:
Name: Prof. Dr. Raad Abdul Gani Basheer Alsanjari
Date: / /2022

بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

﴿ يَرۡفَعِ ٱللَّهُ ٱلَّذِينَ ءَامَنُواْ مِنكُمۡ وَٱلَّذِينَ أُوتُواْ ٱلۡعِلۡمَ دَرَجَٰتٖۚ وَٱللَّهُ بِمَا تَعۡمَلُونَ خَبِيرٞ ١١ ﴾

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DEDICATION

To

My Beloved husband, Beloved Parents and My Beloved Children

IN APPRECIATION OF THEIR CONTINOUS HELP, SUPPORT,

DOA'A AND

LOVE

Abstract

The importance of bone marrow lies not only in providing the body with different blood cells, but rather in its ability to provide the body with renewable stem cells involved in tissue regeneration or stem cell transplantation as a treatment for many diseases. Bone marrow cells are regulated by many intrinsic factors, the most important of which are heat shock proteins and antioxidant enzymes that work together within a micro-environment to maintain the functioning of these cells during lifestyle. The aim of the study was to look into the relationship between gene expression profiling in healthy rats' bone marrow hematopoietic cells through the study of gene expression of certain heat shock protiens Hsps and antioxidant enzymes, and evaluate the bone marrow cytology within first three months old. A total healthy 90 neonate pups were employed throughout the study, divided into three groups according to study design thirty pups each. Group 1 pups were kept till 1 month old, group 2 pups were kept till two months and the third group were left till three months old. When the rats reached the prescribed age, bone marrow samples were taken after euthanized the animas by physical method of euthanasia via cervical dislocation of unanesthetized animals. Bone marrow samples were collected from the femur and tibia. Bone marrow smears were made and stained with May Grunwald Giemsa stain a MGG stain to evaluate the bone marrow cytology. The samples were collected every 5 samples in an Eppendorf test tube, 6 tubes for each group, and kept in deep freezing at -80°C for studying the gene expression of selected genes. The results of bone marrow smears showed different stages of haematopoiesis including erythroid, myeloid, and megakaryocyte cell lines production. Moreover, the morphological evaluation of haematopoietic cells revealed all different types of progenitors

and immature cells of both erythroid and myeloid cells and their counts were varied with age progress.

Moreover, the gene expression of Hsps and antioxidant enzymes showed constant regulation throughout the study except Hsps90α revealed down-regulation at age 3 months compared to first 2 months; antioxidant enzyme GPX1 expression revealed up-regulation at 2, and 3 months old compare to first month of age. In addition to that the fold change of Hsp90β was exhibited an increase in fold change throughout the study period, while Hsp90α was exhibited increase in fold change at 3 months old. The antioxidants enzymes also were showed increase in their fold change throughout the study period.

The relationship between studied genes revealed direct strong relationship between SOD3 and Hsp90β and strong inverse correlation between GPX1 expression and Hsp90α only. On the other hand, other genes showed mild non-significant correlation. Meanwhile, to look into the relationship between gene expression profiling and healthy bone marrow hematopoietic cells was interesting through their correlation among them with independence manner according to the type of haematopoietic cells and certain genes.

The study concludes that bone marrow cells differentiation and proliferation are related to age progress, haematopoietic cells differentiation and proliferation are regulated by bone marrow microenvironment conditions via Hsps and antioxidant genes. Also, Hsps90α and 90β and antioxidant enzymes CAT, SOD3 and GPX1 are the main genes that regulates the myeloid cell line production and differentiation. Hsps90β and 27 and antioxidant SOD3 are mainly regulates the erythroid cell line production and differentiation and maturation. There is a direct relationship

between Hsp90β and SOD3 and inverse relationship between GPX1 and Hsps90α as noticed. There is no correlation between all antioxidant genes and Hsp27 gene expression during haematopoiesis.

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ALB	Albumin
ARE	Antioxidant response element
BM	Bone marrow
CAT	Catalase
cDNA	Complementary DNA
CP	Ceruloplasmin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic reticulum
ESCs	Embryonic stem cells
FER	Ferritin
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HIFs	Hypoxia inducible factors
HSCs	Haematopoietic stem cells
HSFs	Heat shock transcription factors (),
Hsps	Heat shock proteins
HSR	Heat shock response
LIF	Leukemia inhibitory factor
LTF	Lactoferrin
MAPK	Mitogen-activated protein kinases
MB	Myoglobin
MBPs	Metal binding proteins
mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
MTs	Metallothioneins
NADPH	Nicotinamide adenine dinucleotide phosphate
Nrf	Nuclear erythroid factor
OH^-	Hydroxyl
PHSC	Pluripotent haematopoietic stem cell
Qpcr	Quantitative polymerase chain reaction

qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real time-polymerase chain reaction
SCs	Stem cells
SOD	Superoxide dismutase
TF	Transferrin
TNF	Tumor necrotic factor
UPR	Unfolded protein response
VSELs	Very small embryonic-like stem cell

Chapter one

Introduction

1. Introduction

In the early stages of life, the formation, development, and differentiation of bone marrow cells is a very complex process that requires many cellular factors in very strict micro-environments. Heat shock proteins (Hsps) are the most common factors, and they play a vital role in mesenchymal stem cell (MSC) differentiation (Kishor *et al.*, 2013). Hsps act as molecular chaperones that stabilize intracellular proteins, repair damaged proteins, protect existing proteins from clustering, and mediate the folding of newly translated proteins (Fan, 2012). Cellular stress significantly increases Hsps expression. This response has been observed in various types of stem cells (SCs) such as embryonic stem cells, adult stem cells, and pluripotent to maintain their stemness under certain conditions and stress (Shende *et al*, 2019). The heat shock transcription factors (HSFs) are activated, resulting in increased transcription and translation of Hsps (Gomez-Pastor *et al*, 2017). Interestingly, certain Hsps are abundantly expressed in SCs, conversing with specific transcription factors, and are considered to be essential cell development and function (Shaik *et al.*, 2017). Evidently, many positive and negative signals are involved in the tight regulation of SC self-renewal, distinctions, survival, and aging; notwithstanding, growing evidence indicates that Hsps play an important role in the regulation of various types of SCs (Stolzing *et al*, 2006; Yang, 2018). Growing evidence suggests that adequate stress fosters MSC or progenitor cell distinctions, which induces the expression of HSPs, including small and large molecular weight proteins (Zhang *et al.*, 2016). Shifts in Hsp expression were shown to affect stem cell behavior such as self-renewal, differentiation, sensitivity to environmental

stress, and aging . Naturally, oxidative stress in bone marrow happens as a result of activation of endogenous Reactive Oxygen Species ROS from mitochondrial oxidative metabolism as normal function of cell metabolic processes (Bigarella *et al*, 2014).

Diverse bioactive macromolecules are involved in signal transduction and metabolism during cell proliferation; ROS are produced as a result of these two processes. ROS levels in hematopoietic stem cells (HSCs) vary during different cell cycle states, impacting their physiological processes(Ludin *et al.*, 2014). Besides which, inflated ROS levels in HSCs and MSCs inspire HSC relocation and mobilization. Nonetheless, when ROS levels become abnormally high, HSCs may activate a protective mechanism that impedes self-renewal functions (Chen *et al.*, 2019). Cellular arrest and DNA damage are principally associated with a high level of ROS in cells, urging the initiation of a defensive network to scavenge ROS and uphold redox balance in cells (Weiss and Ito, 2014). To summarize, superoxide dismutase (SOD) converts cumulative O2 molecules in cells to H2O2. In general, H2O2 is toxic to cells; however, catalase and/or glutathione peroxidase (GPX1) will quickly convert it to harmless water (H2O). However, if the cells lack catalase and GPX1 to detoxify H2O2, the remaining H2O2 is converted into even more dangerous OH- ions (Phillips *et al.*, 2012). SOD appears to be a two-edged sword. Evidently, it is well known that some antioxidants and prooxidants rely on the existence of other antioxidant enzymes in cells, such as catalase and GPX1. A new global regulator of cellular antioxidant defense known as Nrf2 was discovered, and to date, a variety of ROS-detoxifying enzymes, including SOD, catalase, GPX1, and thioredoxin, have indeed been noticed to be governed by this regulator as downstream effectors. (Denicola *et al.*, 2011). Inflated ROS

levels have been envisioned as one of the influences of HSC defects in aging mice, which was explored by (Porto *et al.*, 2015), who noticed that cytoplasmic ROS levels in HSCs were considerably greater in the elderly than in the young. "Aging" HSCs displayed high levels of DNA damage as well as high rates of senescence and/or apoptosis, and this massive level of ROS was associated with more differentiated HSCs in old-age mice (Porto *et al.*, 2015). What is more, BM stromal cells acquire more ROS than HSCs, diminishing Bone Marrow BM cellularity greatly. Remarkably, expanded ROS levels adversely affect the ability of BM stromal cells to facilitate HSC reconstitution in aged mice (Khatri *et al.*, 2016). This process is tightly linked to the steady progress of age (Asumda and Chase, 2011).

1.1 The importance of the study

The scientific importance of the topic lies in the key role of the oxidative stress in the development and differentiation of bone marrow cells in relation to cellular antioxidants. As well as its vital role in understanding the cellular pathway of mutations leading to leukemia at young ages and its relationship to some blood diseases.

1.2 Limitations

The current study has important constraints. Some of these constraints may not be strange, such as the fact that it is time-consuming, expensive, and restricts the number of substances and kits that can be used, or that it raises tricky ethical concerns because it harms or kills many animals. Animals really are not simply small humans, and their uses quite often miss the most positive associations of bone marrow senescence in humans.

1.3 Important objective

The study's task is to identify the association between heat shock protein and certain antioxidants in bone marrow cells in rats during their first three months of life using a rat model.;

1. Studying the bone marrow cytology during first three months old.

2. Studying the gene expression of heat shock proteins and antioxidant enzymes in bone marrow cells.

3. Finding the relationship between Hsps and antioxidants in bone marrow cells.

Chapter two

Literature review

2. Literature review

The rat (Rattus norvegius) is widely used as a rodent model in physiological and pharmacological studies, that help to understand and figure out multiple pathways which involves different cellular processes. In this chapter, certain topics in relation to BM development links to cellular chaperones and antioxidants will be reviewed.

2.1 Bone marrow cytology

A bone marrow cytology exploration and appraisal is a valuable tool to gauge the hematopoietic system and is diagnostic, prognosis, and tracking of a variety of ailments and diseases. (Raskin and Messick, 2012). The BM cytology undergoes different stages of proliferation and development (Zawar *et al.*, 2018), these stages result in formation and differentiation of multiple cell lines (Bolliger, 2004). 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.

2.1.1 Lineage of erythroid cells

In rats, the erythroid cell line 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. It is an erythroid series stem cell with a large rounded nucleus and basophilic cytoplasm (Moras *et al*, 2017).

Rubriblast undergoes a series of cellular differentiation to form a second cell type, prorubricyte (basophilic erythroblast. It is tinier than a stem cell, and its chromatin is denser. The cytoplasm of RNA-rich cells is basophilic. Hemoglobin content is obscured by basophilia. It divides mitotically, giving rise to rubricytes (Anna Porwit *et al*, 2011). Rubricyte (polychromatophilic erythroblast), which result from the prorubricyte mitosis characterized by small size lesser than the mother cell. A more condensed chromatin with high Hb content and low cytoplasmic basophilia than the mother cell. Identification of polychromatophilic staining owing to the presence of Hb and RNA, which have an affinity for both acid and basic dyes (Romaniuk *et al.*, 2016). The third stage of mitotic division of the rubricytes is the Metarubricyte (normoblast) that characterized by small nucleus and pyknotic and the cytoplasm is increased with hemoglobin content during differentiation of this cell, the nucleus will lose as a final division to produce the last stage of erythroid cell line which is the erythrocyte. Erythrocytes are non-nucleated cells with a circular outline that look dumbbell-shaped from the side due to their biconcave nature and are packed with hemoglobin. Because of the remnant RNA content, immature stages of growth reticulocytes have a diffusely basophilic cytoplasm (Anna Porwit *et al.*, 2011).

2.1.2 Lineage of myeloid/granulocytic cells

Granulocytic precursors in rats are highly homologous to those in other species, but they often have unique features which might make classification difficult. The myeloblast is the initial stage, with a round to oval nucleus, delicately stippled pale chromatin, and one or more visible pale nucleoli (Gupta *et al.*, 2014). The cytoplasm of rat myeloblasts is far

more basophilic than that of other myeloid precursors, of a few primary azurophilic granules. Myeloblasts develop into promyelocytes, which are larger in size. The promyelocytes will develop and differentiate into myelocytes, which are smaller than promyelocytes and have slightly granular cytoplasm similar to that of the fourth stage metamyelocytes, bands, and mature granulocytes, with a predominance of specific secondary granules. In rats, eosinophils possess large and reddish granules, basophils have round and purple granules, and neutrophils include fine and red granules. (De Kleer *et al.*, 2014). It is so difficult to identify and classify the late stages of granulocyte maturation because of granulocytes distinctiveness. Promyelocytes could indeed form ring nuclei, which are differentiated by a small hole in the center of the nucleus. The hole expands and the nucleus shrinks with cell maturation, until only a thin rim of nuclear material remains at the band stage (Patel *et al.*, 2018). Strikingly, the ring form is identified in both neutrophil and eosinophil precursors but not in basophil precursors, and it coexists with far more typical myeloid precursors. At the band stage, the hole expands and the nucleus does become slimmer though only a thin rim of nucleus persists. Rodent neutrophils are found to possess multinuclear indentations, and these criteria enable in the categorization of neutrophils as hyper segmented cells. Myeloid cells are described phenotypically based on nuclear and cytoplasmic criteria, instead of cell shape or size, as erythroid cells are (Ra *et al.*, 2012). The proportion of reproducing myeloid cells (myeloblasts, promyelocytes, and myelocytes) is roughly 11.53-12.11 percent lower than the rate of nonproliferating myeloid cells (metamyelocytes, non-segmented, and segmented granulocytes) seen on BM smear(Reagan *et al.*, 2011)**.**

2.1.3 Lineage of monocytic cells

Monocytes and their precursors are known as low numbers in the BM of healthy rats, unless disease occurs. Monocytes are large cells with nuclei that are round, oval, or convoluted and fine chromatin (lighter than that of most lymphoid cells). Without special stains, it is harder to identify monocyte precursors from early granulocytic cells (Gupta *et al.*, 2014). Cytoplasm is relatively plentiful, light gray-blue in color, and includes few fine azurophilic granules. Macrophages are prevalent residents of rat bone marrow and are limited to small numbers(Gaunt, 2004).

2.1.4 Lineage of lymphoid cells

The bone marrow of rodents is typically rich in lymphocytes, and their cytoplasm may contain azurophilic granules. Lymphoblast and prolymphocytes are available in limited numbers in rat bone marrow and can be difficult to identify from initial myeloid precursors in stained preparations. A lymphoid stem cell, also known as a lymphoblast or lymphoid progenitor cell, is the beginning for a lymphoid cell line. This is a significant turning point because all cells in this line emanates from a lymphoid progenitor cell, whereas all cells in the myeloid line, such as neutrophils, macrophages, and red blood cells, derived from a myeloid progenitor cell. Afterward, the lymphoid progenitor cell (lymphoblast) can differentiate into more specialized cells like B lymphocytes or B cells that produce antibodies. T-cell lymphocytes (Kondo, 2010).

2.1.5 Megakaryocytic cell line

The bulkiest HSCs in rats are megakaryocytes, which are multinucleated with lobulated nuclear mass and therefore are not typically scored but instead qualitatively examined in terms of number, development, and morphology. Their cytoplasm is prevalent, light blue, and tightly packed with fine eosinophilic granules. Younger megakaryocytes have lower nuclear: cytoplasmic proportions, as much basophilic cytoplasm, and fewer nuclei. Megakaryocytes are not exclusive to osteoclasts, which have detached nuclei and less cytoplasm. (Stavnichuk and Komarova, 2021). Mild pleomorphism, evidenced by separated or non-fused nuclei, is an it like of normal rat megakaryocytes. Because of this pleomorphism, a left shift in the megakaryocyte population in rats may be harder to identify or identify than elsewhere species. A normal platelet count and composition in the peripheral blood might well help validate that the pleomorphism observed is normal (Layssol-Lamour *et al.*, 2021).

2.1.6 Nonhematopoietic cells

In recent years, many reports have shown that bone marrow (BM)-derived cells can give rise to differentiated cells of multiple nonhematopoietic organs including the lung, a phenomenon that has become known as bone marrow cell plasticity (Kassmer *et al.*, 2012). BM cytology enriched for many small cells which are not differentiated blood cells, for instance very small embryonic-like stem cell (VSELs) (Kucia *et al.*, 2008; Labedz-Maslowska *et al.*, 2016). Another nonhematopoietic cells found in BM population, for instance, plasma cell which

characterized by oval, deep blue cytoplasm and centric dark dense nucleus (Bene *et al.*, 2021). Mast cells are another type of cell that can still be abundantly found numbers in rat marrow, making up for almost 1% of all nucleated cells (Amani *et al.*, 2019). In rat bone marrow, osteoblasts and osteoclasts can be limited to small numbers.

2.2 Microenvironment of bone marrow

Providing cellular signals that controlling and regulating the production of different hematopoietic cells are essential to maintain homeostasis. The niche which regulates HSCs differentiation and production, is the best description of BM microenvironment (Lucas, 2017). The niche is principle for the keeping haematopoiesis during the life. Under normal circumstances, the niche play a crucial key role in protecting HSCs against sustained and/or overactivation of their growing and development (Fröbel *et al.*, 2021). Haematopoiesis effected negatively and deregulates due to indirect alterations which is occur in their function as consequence to acute and / or chronic cellular stress and these changes happen via the niche. Indeed, these alterations also effect on niche cells through shifting the cellular pathways toward unexpected pattern, for instance, skewing the cellular composition, localization of cell signals that diversely control the function of HSCs and their progeny (Song *et al.*, 2014). Importantly, during acute insults display particularly transitory effects on HSCs, meanwhile, during chronic insults a sustained alterations of the niche will occurs, resulting in HSCs deregulation (Sun *et al.*, 2019). Interestingly, together with extracellular factors, such as chemical and / or physical factors, the BM constitutes a complex niche of different cell types. The balance between HSCs development and

activation is coordinately controlled by cells and these factors. This control pathway by different factors lead to cell determination, proliferation, self-renewal, and finally cell differentiation (Zhao and Li, 2015). Consequently, abnormal regulation of BM niche cells or mis-regulation of the niche is sufficient to influence on cell behavior leading to malignant transformation (Takubo *et al.*, 2017).

2.2.1 The stress of the BM microenvironment and cellular composition

It is crucial to assess the clarification of the homeostatic steady state of the healthy niche in order to know the homeostasis of the BM niche. To constitute the haematopoietic niche, changed the way cells were interlaced with haematopoietic cells, and this specialized cellular network is almost all observed in BM (Wei and Frenette, 2018). Many approaches have been applied to assess the cellular content of the BM under different stress states, in addition to steady state. This approach includes different bulk and single cell analysis, that help in investigating the heterogeneousness in niche cells, via evaluating the cell-cell interaction, communication depending on their receptor pairing and synergy of niche with extracellular matrix (Baccin *et al.*, 2020). Is from the other side, fourteen meta-clusters of cell subgroups outlined by the expression of prohematopoietic factors have been categorized but will aid in the detection of multiple BM cells (Dolgalev and Tikhonova, 2021). Endothelial cells, MSCs, progenitor cells, osteoblasts, chondrocytes, fibroblasts, pericytes, smooth muscle cells, and Schwann cells were determined based on these 14 clusters (Tikhonova *et al.*, 2019; Dolgalev

and Tikhonova, 2021). Interestingly, a fluctuation in stress markers for instance tumor necrotic factor (TNF) level is linked to transient elevation in both melatonin as well as ROS levels in the haematopoietic niche, and this elevation in melatonin necessary for both HSCs self-renewal promotion and protection of stromal niche cells against ROS attack (Pinho and Frenette, 2019). Indeed, this fluctuation between stress and non-stressed haematopoiesis on daily basis indicate the crucial role for antioxidant hormone (melatonin) in protecting the niche from free radical's dependent stress (Calvi and Link, 2015).

2.2.2 The involvement of reactive oxygen species (ROS) in haematopoiesis

Curiously, blood cell formation takes place throughout life, and haematopoiesis, unlike some other divergence processes, must be heavily restricted via the alignment between differentiation and self-renewal in HSCs (Heissig *et al.*, 2005). Furthermore, the significance of HSCs in regenerative process gives an important understanding of HSCs physiology via different pathways. One of these pathways is the contribution of niche to keep low level of ROS in HSCs. Physiologically, enzymatic activity, activated phagocytes as well as mitochondrial respiration by NADPH oxidase system are the main sources of ROS during their normal reactions. Basically, normal cell activity generates ROS which in turn are very harmful to the cellular macromolecules leading to DNA damage which either in turn leads to apoptosis or oncogenesis. ROS levels at resting stem cells are kept low, so promoting their long-term reregulation ability (*Redox Regulation of Stem/Progenitor*

Cells and Bone Marrow Niche, no date). As evidenced in mouse models, ROS production content forces stem cell differentiation to short-term repopulating cells and then to myeloid differentiation. (Jang and Sharkis, 2007).

ROS play an essential part in limiting the stability between self-renewal and differentiation of adult stem cells by restricting their stemness at low levels of ROS, whereas high levels devote them to a restricted lineage (Ito and Suda, 2014). There are two main sources of free radicals in the BM cells, the first is the mitochondria and the second is the membrane Nicotinamide adenine dinucleotide phosphate NADPH oxidase and these radicals are superoxide anion, hydrogen peroxide, and hydroxyl ion (Porto *et al.*, 2015). Indeed, the ROS are formed as consequence to signal transduction and energy metabolism during cell proliferation, and these ROS act as important signalling molecules (Bigarell *et al.*, 2014). During cell cycle states of HSCs, ROS levels oscillate and this oscillation play a key role via affecting on HSCs motility, proliferation, differentiation and repopulation potential. Moreover, elevating of ROS in both HSCs and MSCs promote their migration and mobilization. In addition to that, ROS levels fluctuate during light and dark in HSCs and progenitor cells via particular mechanisms, which result in differentiation and replenishment of both HSCs and MSCs with mature blood cells at day and replenishment of progenitors at night (Golan *et al.*, 2018). Interestingly, an abnormal overproduction of free radicals in HSCs push these cells to shut-down self-renewal function as protective mechanism. Subsequently, to elevation of ROS levels cell cycle arrest and cellular DNA damage will occur and if the damage is too sever to be repaired, the cells may undergo

programmed cell death and/or senescence (*Redox Regulation of Stem/Progenitor Cells and Bone Marrow Niche*, no date). As result of this elevation in ROS, scavenging ROS is arisen as defense mechanism to maintain redox balance in cells. For instance, H_2O_2 was firstly formed from conversion of accumulated oxygen radicals in cells by superoxide dismutase (SOD), since it is very toxic to the cells, it is immediately transformed to nontoxic water (H_2O) by both catalase (CAT) and glutathione peroxidase (GPX), the resting H_2O_2 converted into more toxic OH^-radical (Ludin *et al.*, 2014). Furthermore, OH^- formation and accumulation in cells in turn attack cellular large molecules such as lipids, proteins, and nuclear acids leading to cellular changes in their functions due to cellular destruction of these molecules (Kobayashi and Suda, 2012). Accordingly, detoxifying ROS needs reciprocal group of redox modulating enzymes, for instance, SOD which have double-side sword, one as antioxidant and other as potent prooxidant depending on the existence of other antioxidant enzymes like CAT, GPX and the newly cellular antioxidant regulator Nrf_2 in cells (Denicola *et al.*, 2011). Knowledgeable, a collection of antioxidant enzymes has been controlled by Nef2/Keap1 pathway such as SOD, CAT, GPX and thioredoxin (Ludin *et al.*, 2014). Overall, ROS play a crucial key role in HSCs regulation, either via low levels which help to maintain their stemness or via normal levels which required for promoting HSCs proliferation and differentiation. Nonetheless, an abnormal production of ROS can impact on DNA of HSCs leading to damage it which finally lead to reduced colony formation, and impaired proliferative capacity of these cells (Shinohara *et al.*, 2014; Rönn *et al.*, 2017).

2.2.3 Role of other stress intrinsic factor in BM cell differentiation

In overall, stem cells nest in the BM in specific microenvironments that have an environment for the stem cells to remain dormant. Homogenous progenitor cells can be prompted to reproduce and differentiate by their niche (Bigarella *et al.*, 2014). The progenitors are presence in the neighborhood of BM stromal, haematopoietic, myeloid cells and as well as neurons, which work together to produce chemokines, lipid factors to keep a physiological pool of precursor cells (Wright *et al.*, 2001). Many more stress intrinsic factors or stress-related factors include the low-oxygen stem cell niche (stem cell hypoxia), which includes the endosteal niche, which consists quiescent HSCs, as well as the more oxygenated vascular niche, which includes progenitor cells and distinctive hematopoietic progenitors (Parmar *et al.*, 2007). By offering long-term protection against oxidative stress, niche hypoxia confines H_2O_2 production. As a consequence, niche hypoxia plays a vital role in stem cell maintenance, differentiation, and self-renewal (Nikolsky and Serebrovska, 2010). Hypoxia is the characteristic of BM niche, which has principle character in recirculation and extra structural functioning of HSCs (Zhang and Sadek, 2014). Exposure time to different hypoxia levels in HSCs niche play a contradictory role in cell development via different intrinsic factors, such as transcription factors and cytokines clearly which influence these processes (Szade *et al.*, 2018). Hypoxia inducible factors (HIFs) are linked with other factors that regulating HSCs and progenitor cell behaviour, which act as molecular structure for the hypoxic regulation of cell fate (Takubo *et al.*, 2010). At

the cellular and systemic levels, four types of HIFs cooperate with cellular transcriptional regulators; these HIFs are comprised entirely of regulatory -subunits (HIF-1, HIF-2, and HIF-3) and indicative -subunits (HIF) (Kietzmann *et al.*, 2016). Distribution of HSCs and their proliferating cells are regulated by PO_2 gradient and hypoxic niches. This hypoxic niche was exhibited to limit stem cells self-renewing and induce their early differentiation in vitro conditions (Beerman *et al.*, 2017). These effects are mediated by down-regulation of leukemia inhibitory factor (LIF) signalling pathways. Interestingly, HIF-1α expression leads to inhibit LIF receptor via binding directly with LIF receptor promoter triggering HSCs differentiation via the HIF-1α-mediated suppression of LIF-STAT3 signalling (Huang *et al.*, 2018). Indeed, hypoxia in HSCs activates signalling transduction system via HIF-1 and HIF-3 were expressed in HSCs, which edict cellular metabolism (Takubo *et al.*, 2010). While HIF-2 was expressed throughout hematopoiesis, and the role of HIF-3 in the primitive hematopoietic system is unspecified, this has been shown to be a transcriptional target for HIF-1, it has been shown to play a prominent part in stem cell survival (Wheaton and Chandel, 2011). In HSCs HIF-1α expression or activation induce glycolysis led to inhibit ROS formation rather than mitochondrial respiration due to shitting the cellular metabolism. In addition, NADPH oxidase activity was shown reduced under hypoxia which affecting on ROS production too (Gu and Jun, 2018). Therefore, ROS generators in the cell may influence by hypoxia and / or in coordination with low nutrient via affecting on metabolic activity of progenitor cells (Urao and Ushio-Fukai, 2013). Amusingly, cells in the BM niche govern ROS levels in stem cells in controlling and alignment ROS content in primitive cells.

2.3 Heat shock proteins' roles in BM cytology

Stress at the cellular level induce different defense process pathways to protect the cells and keep, maintain and perform their physiological function. One of these pathways is the induction of heat shock proteins (HSPs) or heat shock response (HSR) which are known also cellular chaperons. Pluripotent self-renewal is a sophisticated regulatory process that is likely multifactorial, including transcription factors, which are a critical regulatory protein in both human and mouse self-renewal (Mitsui *et al.*, 2003). The transcription factors in the SC are working together via upregulation and/or down regulation processes. Nonetheless, cellular chaperones play a contributing role in transcriptional regulatory and signalling networks in differentiated HSCs via regulating their protein conformational and protein complexation state (Korcsmáros *et al.*, 2007). Accordingly, these chaperones are vital for keeping of cellular signal fidelity. Intramolecular interactions mediated by certain Hsp as chaperones and these interactions may folding event and /or intracellular signalling, in addition to protein refolding/degradation (Shende *et al.*, 2019). Distinct Hsp govern and mediates chaperon function in the SC by taking actions as co-chaperones to augment chaperone action. Hsp40, for illustration, improves Hsp70 ATPase activity, and other proteins enable indirect communication within signaling pathways, such as Hsp70/Hsp90 mobilization protein, which behaves as a crosslink between different chaperone systems (Buchner and Allewell, 2019).

Varying gene transcription factors, such as Nanog, Oct4, Sox2, and STAT3, serve an important in SC regeneration and distinctions, and act as a key regulatory protein in both human and mouse embryonic stem

cell self-renewal (ESCs). For illustration, STAT3 serves as an important for the self-renewal of mouse ESCs but not human ESCs, whilst Nanog expression was found in both human and mouse ESCs (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). Surprisingly, gene transcription factors that interact with precise Hsps are known to be involved cell development and function via upstream regulatory oversight and high expressed Hsps. For illustration, stress-induced Hsp90 and comprising expressed Hsp90. Hsp90 is entirely responsible for the folding and maturation of encoding regulators and signal transducers (Sreedhar *et al.*, 2004). During haematopoiesis at the time of differentiation of progenitor cells to different haematopoietic cell lineages via involving of Hsps that, activate protective mechanism of distinct transcription factors. This activation has the ability of direct differentiation processes toward new lineage in immature HSCs (Saretzki *et al.*, 2008). It has been demonstrated that Hsp70 expression in HSCs prevent the inactivation of GATA-1 via activation of caspase mediated proteolysis, which in turn (Hsp70) indirectly prompt erythropoiesis (Ribeil *et al.*, 2006). What's more, Hsp70 endorses cytokine-mediated HSC survival while adversely affecting the consistency of the pro-apoptotic Bim gene, restricting apoptosis in hematopoiesis and leukemogenesis (Matsui *et al.*, 2007). Moreover, activation and proliferation of myeloid progenitor cells involve expression of both Hsp27 and Hsp60 via activation of monocyte-macrophage receptors. Correspondingly, the differentiation processes of blood derived HSCs from umbilical cord. In summary, all Hsps act as stimulating and/or impeding in mechanisms underlying SC proliferation and differentiation events.

2.4 Antioxidant defense mechanisms

As a response to oxidative stress, specific enzymatic and non-enzymatic substances endogenously found within the biological system in low concentrations to overcome oxidizable substrates or free radicals that already produced as waste products at cellular level, also there are exogenous substances at as antioxidants. It can be hard to differentiate between enzymatic and non-enzymatic antioxidants since some non-enzymatic antioxidants behave as co-enzymes or substrates to that enzyme, interfering including its action in the enzymatic system (Nimse and Pal, 2015).

2.4.1 Types of enzymatic antioxidants

There really are diverse enzymatic antioxidants existing in the biological process, like superoxide dismutase (SOD), an enzyme that facilitates in the rundown of possibly hazardous oxygen molecules in cells, and this process might very well avert cellular injury. The first line of enzymatic antioxidants is the SOD, which also known as detoxification enzyme and the most potent endogenous antioxidant (Ighodaro and Akinloye, 2018). SOD is indeed identified as a metalloenzyme due to and need for metals as coenzymes to do the its function, such as Fe, Cu, Mn, and Zn. SOD did work by catalyzing the dismutation of two molecules of superoxide anion to H_2O_2 and a molecule of oxygen, producing the possibly dangerous radicle less unsafe (Roberts *et al.*, 2007). Indeed, SOD playing a vital role in cellular health as well as its role in protecting cellular system from overproduction of free radical and other cellular harmful agents which promote aging and/or

apoptosis. In general, progress in age leading to elevation in free radical as consequence to increase of catabolic processes as well as, decline of SOD levels (Irato and Santovito, 2021).

Catalase (CAT) is another enzymatic antioxidant discovered in all oxygen-consuming tissues which is distinguished via the use of Fe or Mn as a coenzyme. Deteriorated H2O2 catalyzes the formation of water and molecular oxygen, having completed the detoxification process initiated by SOD (Chelikani, Fita and Loewen, 2004). CAT is a key biological antioxidant which is viewed as a first-line defense enzyme due to its ability to effeciently reduce H2O2 concentration in cells (Irato and Santovito, 2021). During mutagenesis and inflammation CAT playing a crucial role due to its expression via CAT gene as a response to induce free radical production. As well as elevation of CAT was noticed during suppression of apoptosis, which is linked to oxidative stress conditions (Sandstrom and Buttke, 1993).

The third intracellular enzymatic natural is glutathione peroxidases (GPX), that playing a vital role of inhibiting lipid peroxidation process via breaking down hydrogen peroxides to water through removing oxygen molecule in mitochondria to protect cells from oxidative stress (Baek *et al.*, 2007). Selenocysteine peroxidase is the other name of GPX due to its activity, which depend on selenium as cofactor (Wehrle *et al.*, 2019). Importantly, GPX is divided into four types in human; GPX 1, 2, 3, and 4 according to specific regulating genes. For instance, GPX1, 4 are found in all cells, GPX2 found in intestine, while GPX3 is primary located in the kidneys as well as present in extracellular fluids as glycoprotein (Bae *et al.*, 2009). In rodents, there are another two designated GPX, which 5, and 6 that are characterized by their

independency of selenium to perform their activity in scavenging hydrogen peroxide (Baek *et al.*, 2005). In general, all types of antioxidants act in the cells via three mechanisms; preventive action via preventing free radical's interactions or their by-products with intracellular molecules. Other mechanism is inactivation of the free radicals and their derivatives via repairing and/or erases damaged structure. The last mechanism is repairing system via combining interruption into free radical oxidation reaction (Frei, 1994). This pathway could indeed repair broken DNA and proteins, compete oxidized lipids, cease the chain propagation of peroxyl lipid radicals, and eventually fix broken cell membranes and molecules (Muftuoglu *et al.*, 2014).

2.4.2 Types of non-enzymatic antioxidants

Free radical chain reactions intercept and terminate via the activity of non-enzymatic antioxidants, which naturally present in the cells endogenous antioxidants as well as exogenous antioxidants. For instance, of these non-enzymatic endogenous antioxidants are vitamin A, C, E, glutathione, uric acid, melatonin, and bilirubin. Some of these antioxidants are present in the cytoplasmic matrix and/or cytosol due to their water-soluble characteristic, the other type of antioxidants which are lipophilic substance, they are present in cell membrane (Mirończuk-Chodakowska *et al.*, 2018). While exogenous antioxidants include carotenoids, flavonoids, theaflavin, curcumin, and plant polyphenols (Ziad Moussa, 2020). Furthermore, there are more endogenous non-enzymatic antioxidants known as metal binding proteins (MBPs), for instance, extra- and intracellular proteins, like albumin (ALB),

ceruloplasmin (CP), metallothioneins (MTs), ferritin (FER), myoglobin (MB), transferrin (TF) and lactoferrin (LTF). The role of MBPs for example is playing as plasma antioxidant capacity via ability to bind metal ions such as Cu^{2+} and Fe^{2+} which act as pro-oxidant doing their action through Fenton reaction by catalyzing production of ROS, these MBPs act as true scavengers of ROS (Valko *et al.*, 2006; Birben *et al.*, 2012; Taverna *et al.*, 2013). Glutathione (GSH) in its two forms, reduced and oxidized are another non-enzymatic soluble antioxidant which present in high concentration in the cytoplasm, mitochondria and nucleus. Interestingly, around 99% of GSH is present in RBC, while 1% of GSH present in the plasma which considered as intracellular oxidized glutathione (Lushchak, 2012). Throughout non-enzymatic reactions, GSH as an antioxidant drops or erodes ROS; GSH reproduces other small molecule antioxidants such as vitamin C and vitamin E, which are participated in the restoration DNA, lipids, and proteins in peroxidation processes in able to preserve protein sulphydryl groups in a reduced state (Rahman, 2007; Lushchak, 2012).

In fact, oxidative stress is vital in the onset of cell growth and division; nevertheless, reactive species generated in the process among several metabolic processes throughout cell proliferation can indeed be injurious to the cells. Even as enzymatic antioxidants are assumed to be the first line of defense against oxidative stress, non-enzymatic antioxidants are presumed to be the second line of defense against free radicals due to their fast downregulation of radicals and oxidants (Rahman, 2007).

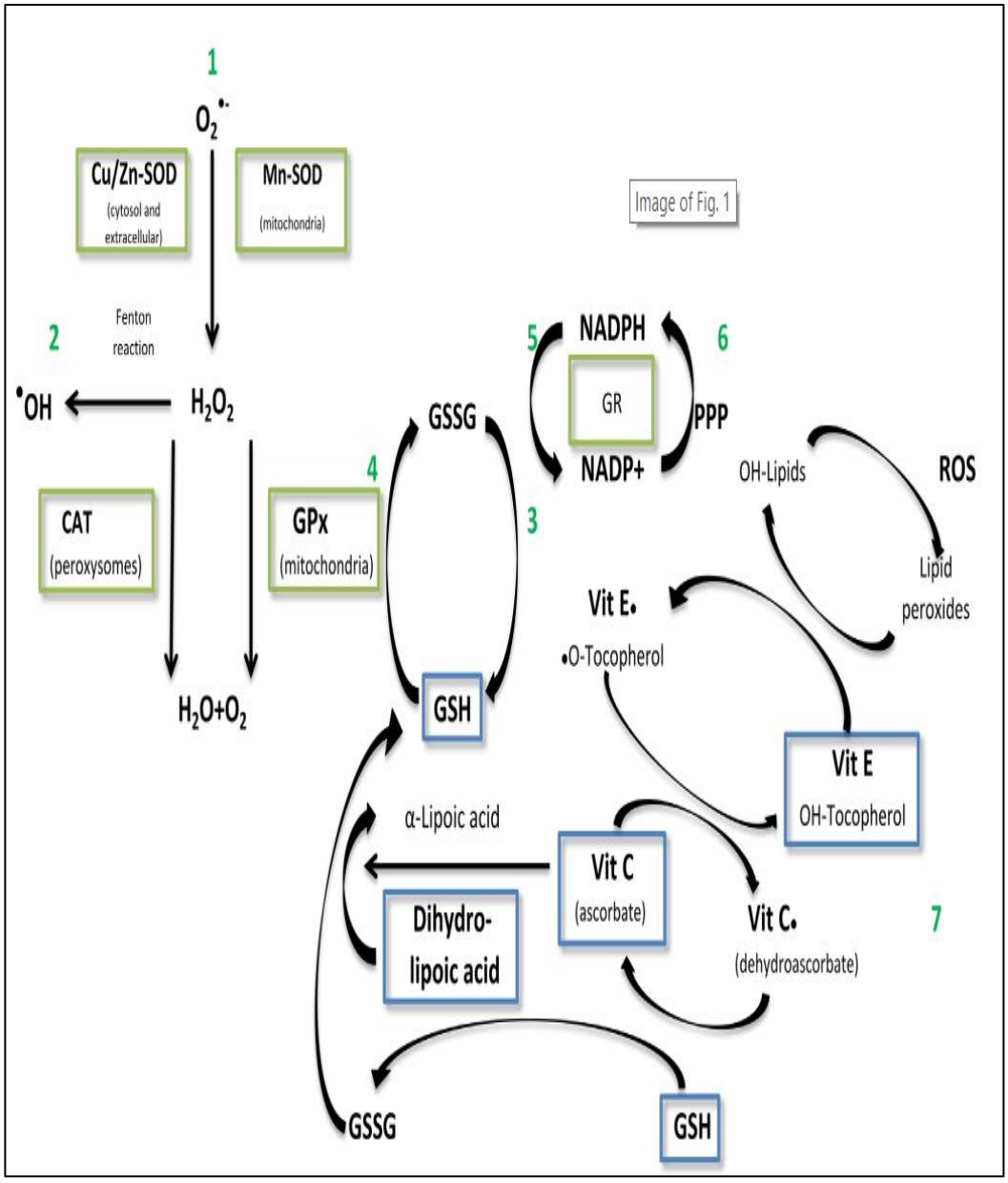


Figure 2.1 The mechanism of action of antioxidants (Hunyadi, 2019).

1. A single-electron reduction of oxygen tends to produce superoxide radical. Superoxide radical covalently an electron in a catalysed by superoxide dismutase (Cu/Zn-SOD or Mn-SOD), leading to the creation of H_2O_2. Two enzymes, catalase (CAT) and glutathione peroxidase, are implicated in the lowering of H_2O_2 to water and oxygen (GPx).
2. H_2O_2 is converted to hydroxyl radical (HO-) in the Fenton's reaction, which is provoked by transition metals, and then partakes in free radical chain reactions.
3. Cos of the involvement of the thiol group of cysteine, reduced glutathione (GSH) responds with free radicals of proteins or other macromolecules, rebuilding them to the reduced form.
4. In the pathway involving by glutathione peroxide, H_2O_2 is simplified by reduced glutathione.
5. Glutathione disulphide is simplified by glutathione reductase (GR) the with aid of NADPH hydrogen, which then is oxidized to NADP+.
6. The first oxidative step of the pentose phosphate pathway (PPP) produces NADPH. Throughout this step, glucose-6-phosphate is hydrolyzed to ribulose 5-phosphate by glucose-6-phosphate dehydrogenase, even as two molecules of NADP+ are reduced to NADPH.
7. Vitamin C and a-lipoic acid aid in the restoration of GSSG back into GSH. A hydrogen donor As a hydrogen donor, vitamin E works to remove lipid peroxides and ends oxidative chain reactions. Vitamin C and glutathione can recycle unoxidized vitamin E (Jat, 2016).

2.5 Role of antioxidants in homeostasis of bone marrow niche

Only HSCs are capable of delivering all blood lineages thru out life, and this process is strictly controlled by niche, where it restricts the quiescence, expansion, and differentiation of HSCs (Wei and Frenette, 2018). Cellular pathway signals within the niche, such as hypoxia and stress, serve an important in niche autoregulation, but it has an influence on HSC proliferation and differentiation via HSC specific stimuli to stress conditions (Warr *et al.*, 2011). Whenever previously stated, the buildup of ROS in HSCs plays a major role in preserving the stemness of self-renewal cells differentiation to myeloid lineage in rodents, while also enforce the stability for both self-renewal and differentiation of adult stem cells thru the rules to pluripotency of these cells at poor levels of ROS, while elevated levels dedicate them to a restrained lineage. Pertinently, ROS including superoxide anion, hydrogen peroxide, and hydroxyl radical, that could induce cell injury, are physiological derivatives of respiratory chain reaction processes derived as a cellular metabolism end-product via oxidative reactions of mitochondria. What's more, like a defensive response, cells must inactivate inordinate Ros levels via cellular antioxidants that include both enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidases (GPXs), thioredoxin (Trx) as well as non-enzymatic antioxidants that cumulatively minimize oxidative state (Matés, 2000).

Notably, HSCs and niche preservation against ROS overcapacity are inextricably linked to the hypoxia environment vital link with far more effective HSCs. A sequence of metabolic pathways use oxygen

throughout cell proliferation, and as a result of activities, ROS are developed and released, potentially cause genotoxicity and protein misfolding (Huang *et al.*, 2019). Moreover, ROS more than leading to cellular damage also activates the p38 MAPK pathway, that effect negatively on molecule expression and enhance cell cycle progression. As a consequence to activation of p38 MAPK pathway, certain enzymatic antioxidants expressed and protect the cell from damage as well as play an crucial role in repair system of the cell (Gupta, Karpatkin and Basch, 2006). Ultimately, hypoxia in the BM niche defends HSCs from ROS-induced damage, which really is essential to preserving HSC self-renewal capacity and avoiding stem cell exhaustion, even though higher levels of oxygen may ease progenitor proliferation (Urao *et al.*, 2013). Nonetheless, lot remains to be elucidated about the role of antioxidants in HSC expansion, distinctions, and niche, including biomolecules on HSCs and the bioactivities of adult haemangioblasts in replenishing the HSC pool.

Chapter three

Materials and Methods

3. Materials and Methods

3.1 Animals

The scientific committee of the department of physiology, pharmacology, and Biochemistry/Veterinary Medicine College/University of Mosul approved the study, which was performed in accordance with our scientific guidelines for animal studies.

The female rats were kept in plexiglas boxes for adaptation for two weeks under standard laboratory conditions. Fifteen randomly healthy female rats were mixed with healthy male rats for getting pups that employed in the study. First-time-pregnant rats were housed in terms of humidity, temperature, and light. A total 90 pups were involved throughout the study according to study design. Growing pups were kept with their moms till the weaning day, then transferred to large breeding cages and 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. Throughout the study, rats were given free access to clean tap water and standard rat pellets.

3.2 Study design

A randomly selected ninety pups from normal, and healthy female rats were chosen at the weaning day for the study, and distributed in three large breeding cages. Thirty pups were kept in each cage, the study work flow was designed as follow. One month old pups were chosen from weaned pups at day 28 and left for another two days, then samples of

bone marrow. 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.

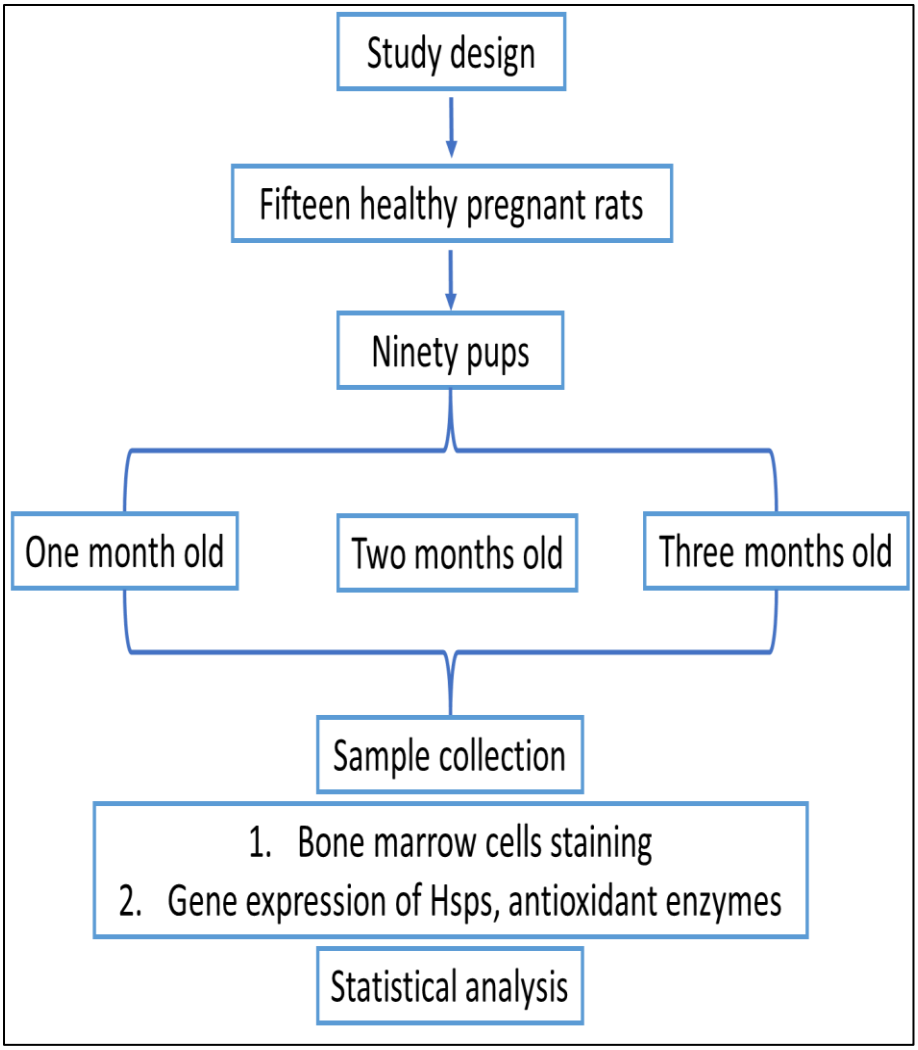


Figure 3.1: study design

3.3 Bone marrow collection

According to the specified study times, 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 (Shomer *et al.*, 2020). The procedure for euthanasia used in animal trials at the Veterinary Medicine College/University of Mosul was approved by the Animal Ethics Committee (IAEC). In summary, the euthanized animal was back-side extended, shaved, aseptically ready for surgical incision on the lateral side of the thigh, and both the tibia and femur were exteriorized just after muscular and tendinous attachments were separated. With a scissor, the metaphyseal region of both bones was cut and a needle was inserted into the medullary cavity, the bone marrow was flushed out using 5 ml of PBS containing 3% EDTA in a petri dish, moved to a spin column, then mixed by vortex, filtered by mesh, and flushed out bone marrow was centrifuged. The supernatant was decanted and the sedimented cells were mixed well by pipetting and used for bone marrow smear and then kept in deep freeze at -80º C for gene expression.

3.4 Bone marrow smear

The reason for using EDTA is to prevent clotting of the sample and to facilitate the preparation of smear. In short, 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 laying a second slide straight on top of the first, the bone marrow can distribute. 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 (Raskin and Messick, 2012).

3.5 Staining protocol and cell counting

A modified staining protocol of (Zawar *et al.*, 2018). Briefly, after being the slide prepared and the smear done, the next step was the staining which was run as follow;

1. carefully immerse the slides in absolute methanol for 10-15 minutes for fixation.
2. The May Grunwald stain and phosphate buffer pH 6.8 were developed next. In a Coplin jar containing May-Grunwald solution, submerge the slides for 20 minutes.
3. After preparing a 1:10 dilution of Giemsa stain with phosphate buffer pH 6.8, place the slides in a jar of buffer solution with a pH of 6.8 for 5 minutes.
4. The slides should then be immersed in Giemsa R solution for 20 minutes before being washed with pH-neutral water for 30 seconds.
5. After 20 minutes, pour off the stain and rinse the slides under running water. Then, for another 15 minutes, dip the slides in a jar of Giemsa mixture and stain.
6. Pouring off the stain and rinsing the slides under running water.
7. Then the last next step was air drying the slides Placing the long cover slip on the area of interest was the last step before reading the slides under 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.

3.6 mRNA expression of Hsps and certain antioxidant enzymes

3.6.1 RNA extraction and cDNA transcription protocol.

Frozen bone marrow samples at -80° C were used for isolation of total RNA using SV Total RNA Isolation System, (Promega, USA). The protocol run according the manufacturer instructions with modification. Briefly, every 5 bone marrow samples of each group were mixed together with 5ml neutralized PBS and centrifuged at 13000 rpm up to minutes, the supernatant was discarded to get BM cells' pellet. The pellet was suspended with 1ml PBS and used for total RNA isolation by adding 175µl of RNA Lysis Buffer to the suspended cells in 2 ml tube, dispersing the pellet and mixing well by vortexing. The homogenate then was incubated with 350µl of RNA Dilution Buffer (blue). Invert the tube four times to mix. The specimens then were placed in a 70°C water bath for 3 minutes prior to getting centrifuged at 13000 rpm for 10 minutes. Pipetting was used to transferring the cleared lysate solution to a fresh microcentrifuge tube thus preventing disturbing the pelleted trash. Afters that, 200µl 95 percent ethanol was pipetted into the cleaned lysate and stirred four times. The mixture was taken to the Spin Column Assembly and centrifuged for one minute at 13000 rpm. The Spin Basket was withdrawn from the Spin Column Assembly, and the liquid in the

Collection Tube was dumped. The Spin Basket was then added back into the collecting tube, 600µl of RNA Wash Solution was poured to the Spin Column Assembly from being diluted with ethanol, and centrifuged at 13000 rpm for 1 minute. Empty the collection tube and place it in a rack and 50µl of freshly prepared DNase was added directly to the membrane inside the spin basket with making sure that the solution was in contact with and thoroughly covering the membrane. The spin basket was incubated for 15 minutes. After this incubation, 200µl of DNase Stop Solution was added to the spin basket, and centrifuged at 13000 rpm for 1 minute without emptying the collection tube. 600µl RNA Wash Solution was added and centrifuged at13000 rpm for 1 minute. After draining the collection tube, 250µl of RNA Wash Solution was added and centrifuged at high speed for 2 minutes. The final phase in RNA isolation would have been to transfer the spin basket out from collection tube to the 1.5 ml Elution Tube, subsequently pour 100µl Nuclease-Free Water to the membrane and sufficiently cover the surface with water. The Spin Basket Assemblies then were set in the centrifuge with the Elution Tube lids facing out and cycled at 13000 for 1 minute. The Spin Basket was retrieved and thrown away. The isolated RNA in the Elution Tube was packaged and stored at -70°C for the cDNA transcription step.

The conversion of RNA to cDNA was done by transforming up to 5µg of total RNA or up to 500ng of RNA into first-strand cDNA through using GoScriptTM Reverse Transcriptase kit technique. In short, the technique was divided into two phases. The first phase was to produce the reverse transcription reaction mix by merging the ingredients of the GoScriptTM Reverse Transcription System in a sterile microcentrifuge tube on ice. Each cDNA synthesis reaction needed 15µl of solution,

which was vortexed softly to mix and stored at -20 before dispensing into the reaction tubes., as follow;

Components	Amount
Nuclease-Free Water (to a final volume of 15µl)	6µl
GoScript™ 5X Reaction Buffer	4.0µl
MgCl2 (final concentration 1.5–5.0mM)	3.0µl
PCR Nucleotide Mix (final concentration 0.5mM each dNTP)	1.0µl
GoScript™ Reverse Transcriptase	1.0µl
Final volume	**15.0µl**

The First-Strand cDNA Polymerization was then equipped and run by adding 15µl aliquots of the reverse transcriptase reaction mix with each reaction tube on ice, then adding 5µl of RNA and primer mix to each reaction for a total reaction volume of 20µl per tube and incubating with a Biometra thermal cycler (Tprofessional® Basic 96, An Analytic Jena Company, Germany). The approach outlined below was being used: 5 minutes priming at 25^{o}C. Reverse transcription at 42°C for up to 53 minutes, followed by 1 minute of RT inactivation at 95^{o}C. Finally, cDNA samples were stored at -20 oC until more processing.

3.6.2 Running RT-qPCR

The genetic information of heat shock proteins and antioxidant enzymes mRNAs was examined using RT-qPCR on a Bioevapeak Real-Time PCR SystemTM Real-Time PCR System (China) through using SYBR green approach on a GoScriptTM Reverse Transcription System.

The contrast Ct strategy has been used to assess relative gene transcription via relative qPCR. The RT-qPCR reaction was carried out in all samples in a total reaction mixture volume of 25µl as follows: 2µl cDNA (templates), 1µl forward primer (20 pmol/l), 1µl reverse primer (20 pmol/l), 12.5µl FastStart Universal SYBR Green Master (Rox), and 8.5µl deionized water. The RT-qPCR cycling conditions were optimized: 35 cycles of annealing at 95^{o}C for one minute, 60^{o}C for one minute, and extension at 72^{o}C for one minute. The initial denaturation took place at 95^{o}C for three minutes. The relative qPCR was performed utilizing Bioevapeak Real-Time qPCR Software (PCR-Q96-5) V. 2022, and findings from each sample were adjusted to βactin expression (housekeeping gene). qPCR was run to analyse the following Hsps (27, 90α, 90β) and antioxidant enzymes (GPX1, CAT, SOD3) mRNAs which supplied by integrated DNA technologies, Singapore.

mRNAs	Sequences
B actin	F 5'-TTG CCC TAG ACT TCG AGC AA-3' R 5'-AGA CTT ACA GTG TGG CCT CC-3'
GPX1	F 5'-CGA CAT CGA ACC CGA TAT AGA-3' R 5'-ATG CCT TAG GGG TTG CTA AGG-3
Catalase	F 5'-CAG CGA CCA GAT GAA GCA-3' R 5'-GGT CAG GAC ATC GGG TTT C-3'
SOD3	F 5'-TGG GAG AGC TTG TCA GGT G-3' R 5'-CAC CAG TAG CAG GTT GCA GA-3'
Hsp 27	F 5'-GAG GAG CTC ACA GTT AAG ACC AA-3' R 5'-TTC ATC CTG CCT TTC TTC GT-3'
Hsp 90α	F 5'-TTT CGT GCG TGC TCA TTC T-3' R 5'-AAG GCA AAG GTT TCG ACC TC-3'
Hsp 90β	F 5'-TGG TGG ATC CTT CAC TGT CC-3' R 5'-TTT CTT CAC CAC CTC CTT GAC-3'

3.7 Statistical analysis

Statistical analyses were performed via IBM SPSS Statistics 22. (SPSS In. Chicago, IL., USA). The mean and standard error (S.E.) of the cell type/duration were used to represent the statistics for cytological assessment and gene expression. One-way analysis of variance (ANOVA) has been used to evaluate quantitative data. A Pearson two-tailed correlation test between Hsps and antioxidant enzymes, as well as between the same parameters and study timeframe, was used to test if variations between study groups were statistically significant when compared to 1-month-old rats. (Corporation, 2011).

Chapter four

The results

4. The results

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

4.1.1 Characterization of healthy rat bone marrow cells

Bone marrow cytologic characterization has been meticulously and comprehensively conducted in accordance with (Figueiredo *et al.*, 2016). The diverse patterns of PHSc that give rise to other blood cells were detectable in the bone marrow slide. Such PHSc are round, non-adherent, possess rounded nuclei, and have a poor cytoplasm-to-nucleus ratio. Furthermore, the examination showed presence of haematopoietic cells which appear as a very impressive painting 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 smears. Figures 4.1, 4.2, 4.3, 4.4 and 4.5.

The haematopoietic cell evaluation revealed different type of cells according to their morphological and staining feature as described in table 4.1 for erythroid cell line production. In this table the erythroid precursors revealed a series of proliferating cells which 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 4.1.

Furthermore, the bone marrow smear revealed another cell line production which is the cells of the granulocytic line. he granulocyte cell

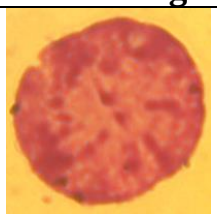
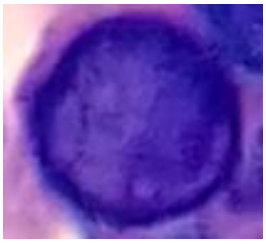
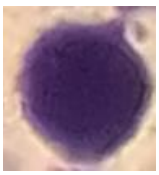
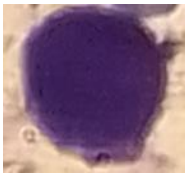
line production showed mature and immature cells in a comparable pattern to the erythroid cell line production, with proliferating cells—myeloblasts, promyelocytes, and myelocytes—occurring to a minor extent than nonproliferating cells—metamyelocytes, nonsegmented, and segmented granulocytes—which are typically seen in healthy rat bone marrow smear as in Table 4.2.

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 is indeed an enormous, multinucleated, granulated, and oddly shaped cell. The sole, enormous, spherical nucleus, delicate chromatin, pale nucleoli, sparse profoundly basophilic cytoplasm, few vacuoles, plus cytoplasmic blebs highly prevalent on the cell's border are characteristic of the megakaryoblast. The promegakaryocyte had two to four distinct nuclei, more packed chromatin, a bigger size than the megakaryoblast, little to no profoundly basophilic cytoplasm, few vacuoles, and cytoplasmic blebs. The developed megakaryocyte became distinguished from the promegakaryocyte by having an enormous multilobulated nucleus with packed chromatin, little to no basophilic cytoplasm, few vacuoles, and cytoplasmic blebs with eosinophilic highly granular cytoplasm. The platelet felt like eosinophilic bits of cytoplasm that have been asymmetrical, Figure 4.6.

Figure 4.7, showed different cell type and shape of monoblast which usually show abundant dark to light-blue cytoplasm with no or a few scattered fine azurophilic granules. The nucleoli are round, oval, or folded, and the nuclear chromatin is fine. There is often a single large nucleolus. Moreover, healthy rats' bone smear revealed different

lymphocytes with large nucleus displayed a distinct monoblast cell type and form from what is typically seen, which is an abundance of dark to light blue cytoplasm with few to no fine azurophilic granules. The nuclear chromatin is tight, and the nucleoli are spherical, elliptical, or twisted. There is frequently just one big nucleolus. Meanwhile, a bone smear of healthy rats showed several cells with big nuclei, figure 4.8.

Table 4.1: Erythroid cell line production in rat bone marrow

Cell type	Cell image	Cytologic description
PHSC		Spherical, quasi blood cells can provide rise to additional blood cells get a small cytoplasm-to-nucleus proportion and a curved nucleus.
Rubriblast		a heightened N/C proportion, a thin margin of intensely basophilic cytoplasm, a spherical central nucleus, frail chromatin, medium-sized pale nucleoli, and an elevated N/C ratio.
Prorubricyte		Narrower than the rubriblast, with such a better N/C ratio, a spherical central nucleus, wider free chromatin, nucleoli that are typically concealed, and cytoplasm that is incredibly basophilic.
Rubricyte	 	Wider than prorubricytes, but varying in dimension from tiny to moderate; spherical core nucleus; densely clustered chromatin; basophilic cytoplasm Another kind occurs that is thinner than prorubricyte.

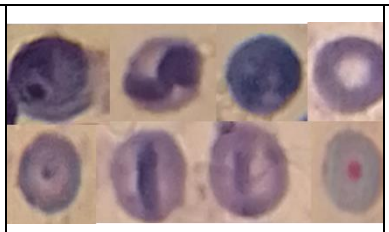
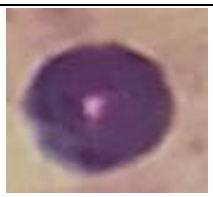
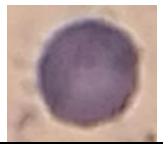
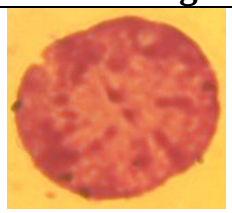
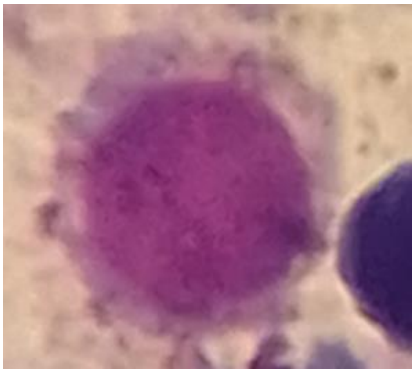
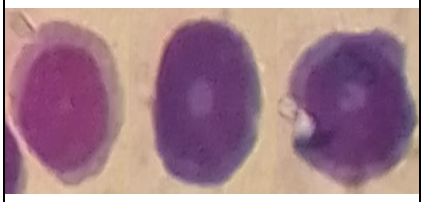
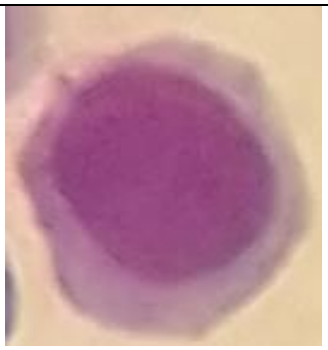
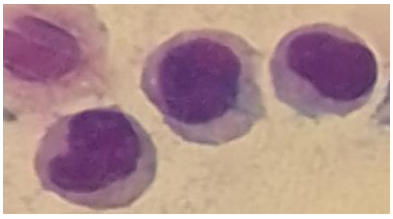
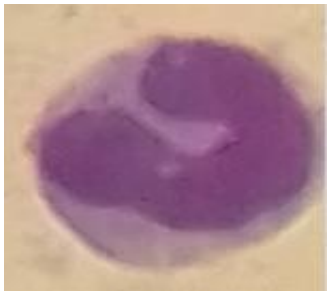
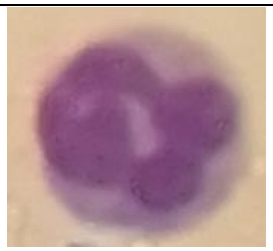
Metarubricyte (nucleated RBC)		Tiny, dense, uniform, pyknotic nucleus; enlarged size to polychromatophilic RBC; polychromatophilic or orthochromic cytoplasm
Polychromatophilic erythrocyte (reticulocyte)		In opposed to fully formed RBCs, the cytoplasm is nonnucleated, murky gray (grayish blue), and substantially thicker (polychromatophilic)
Mature erythrocyte		Nonnucleated cell containing orthochromatic cytoplasm (red-orange)

Table 4.2: Myeloid cell line production in rat bone marrow

Cell type	Cell image	Cytologic description
PHSC		Circular, ou pas blood cells can offer lead to rising blood cells get a small cytoplasm-to-nucleus proportion and a circular nucleus.
Myeloblast		Promyelocyte-like in dimensions or somewhat thinner, with said greatest N/C proportion, a round to circular nucleus, finely mottling chromatin, one to several pallid nucleoli, and somewhat basophilic cytoplasm with little or no basic granules.

Promyelocyte		size is greater or roughly comparable in size to myelocytes, increased N/C proportion, disk - shaped nucleus, flaky to rougher, higher transparent chromatin, inadequate distinguishing nucleoli, or nucleolar rings, sky blue cytoplasm, no identifiable granules, sporadic tiny bright red (azurophilic) basic granules
Myelocyte		wider or comparable to metamyelocytes in dimensions, increased N/C ratios, irregular nucleus that's also round to elliptical, greater porous chromatin, absence of nucleoli, cytoplasm, and specialized granules that are similar to more mature cells
Metamyelocyte		Greater N/C proportion, peanut nucleus, more open chromatin, paucity of nucleoli, cytoplasm, and distinct granules matching more advanced stages, and cells that are slightly bigger than band cells
Band cell		Relatively small as differentiated cells, bent nucleus with straight equal lines and no limits, thinner clustered chromatin, identical to mature cells in respect of cytoplasm and unique granules.

Segmented granulocytes		Structured nucleus, chromatin that is flexibly packed, transparent to moderately basophilic cytoplasm, and the occurrence of such granules

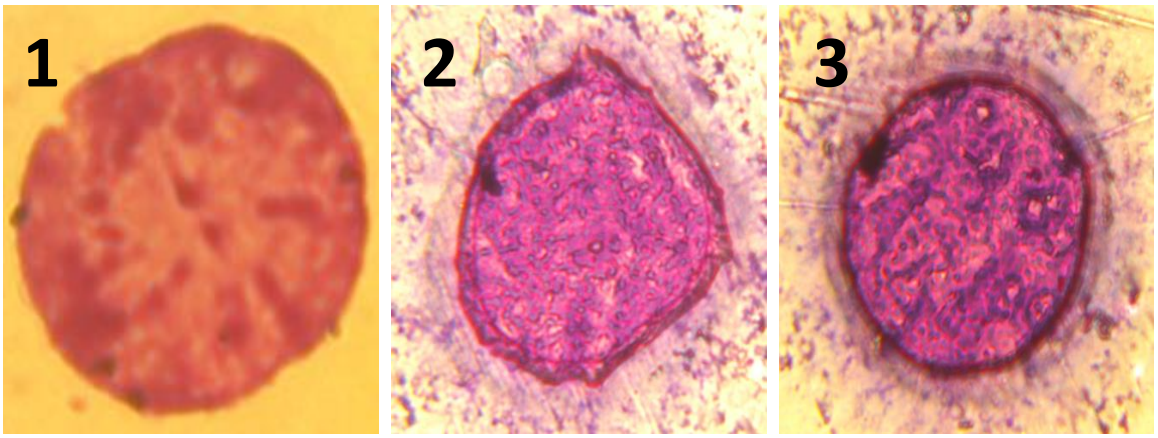


Figure 4.1 Pluripotent Stem cell of rats' bone marrow smear. Higher magnification reveals three different shapes of pluripotent stem cell (1, 2, 3). MGG stain. 100× objective magnification.

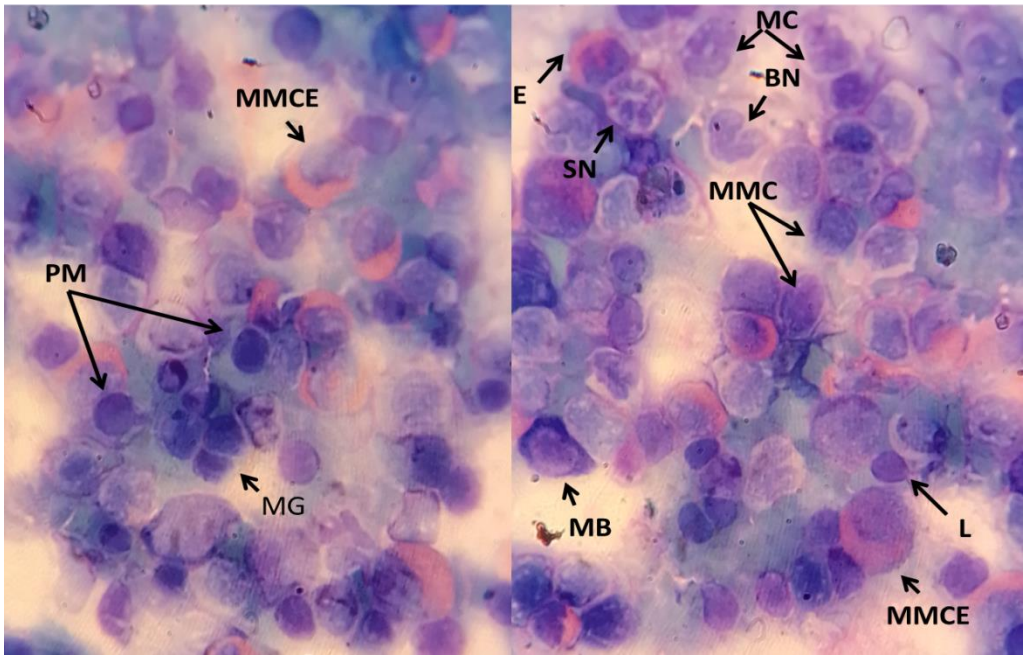


Figure 4.2 Different cells of rats' bone marrow smear. Higher magnification reveals myeloid precursors consisting of myeloblasts (MB), promyelocytes (PM), myelocytes (MC), metamyelocytes (MMC), metamyelocytes eosinophile (MMCE) band neutrophils (BN), and segmented neutrophils (SN), eosinophile (E) as well as megakaryocyte (MG). MGG stain. 40× objective magnification.

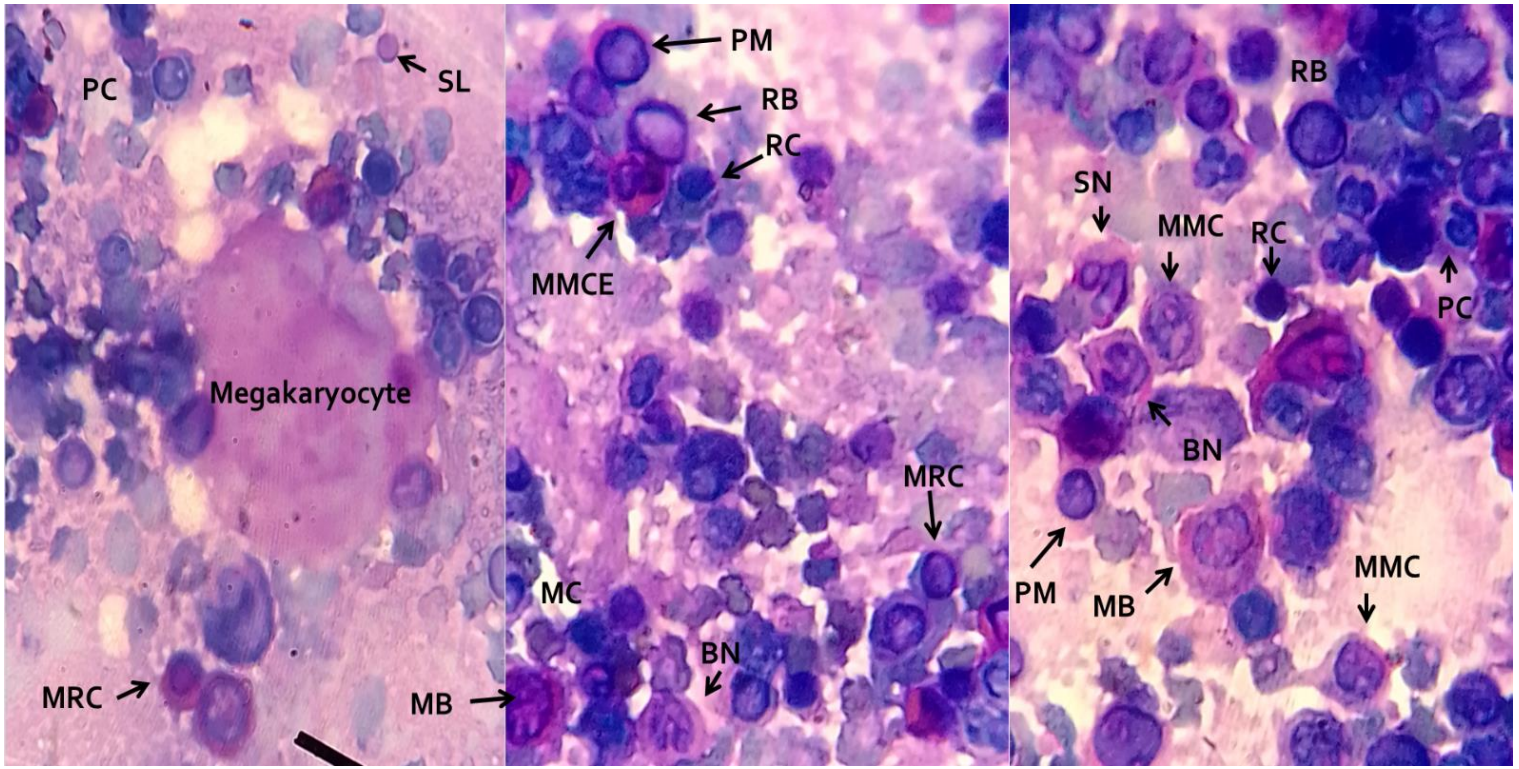


Figure 4.3 Bone marrow smear. Higher magnification indicated the existence of erythropoiesis such as rubriblasts (RB), prorubricytes (PR), rubricytes (RC), and metarubricytes (MRC), as well as myeloid

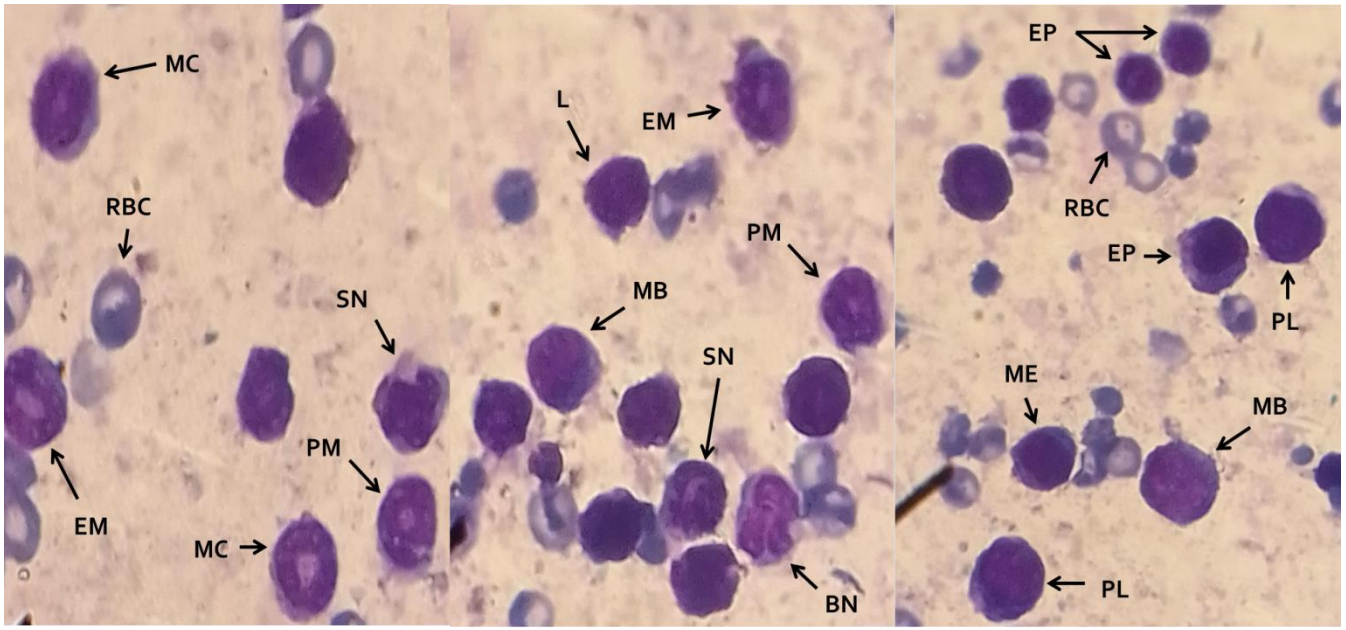


Figure 4.4 Granulocytic progenitor rats' s in normal bone marrow. Higher magnification showed myeloid progenitors consisting of myeloblasts (MB), promyelocytes (PM), early myelocyte (EM), myelocytes (MC), band neutrophils (BN), and segmented neutrophils (SN) and erythroid progenitor (EP), metarubricyte (ME), and prolymphocyte (PL) and lymphocyte (L) are identified. Polychromatophilic cells (PC). MGG stain. 100× objective magnification.

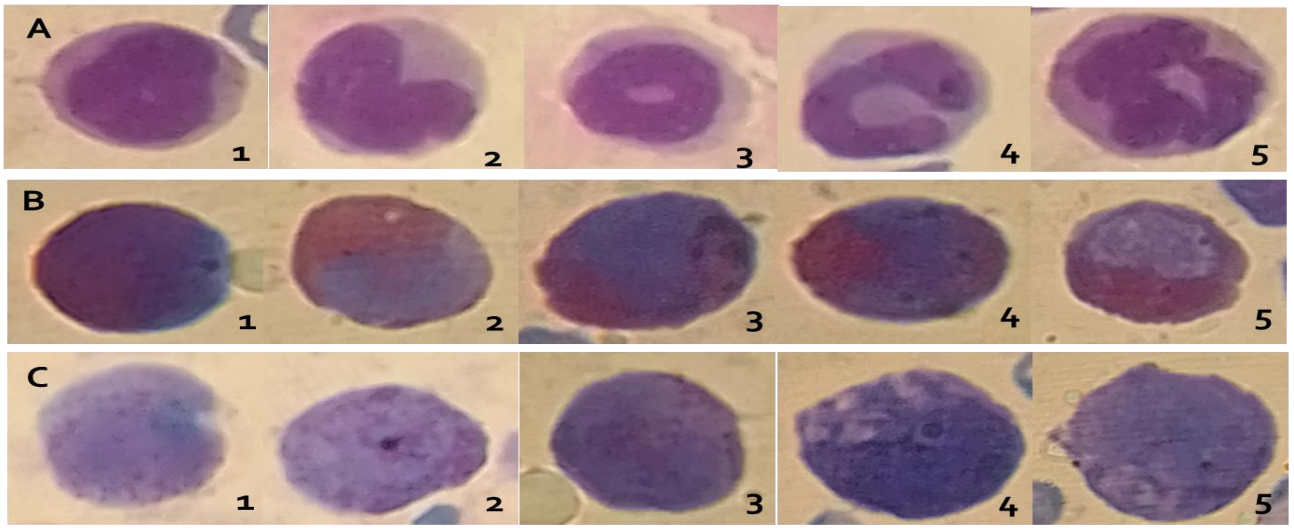


Figure 4.5 Granulocytic series of neutrophils, eosinophils and basophils. Higher magnification reveals myeloid precursors consisting of different cell types, (A) 1. Promyelocyte neutrophile, 2. Early myelocyte, 3. Myelocyte neutrophile, 4. Band neutrophile, 5. Segmented neutrophile. (B) 1. Promyelocyte eosinophile, 2. Early myelocyte eosinophile, 3. Metamyelocyte eosinophile, 4. Late metamyelocyte eosinophile, 5. Eosinophile. (C) 1. Promyelocyte basophile, 2. Early myelocyte basophile, 3. Metamyelocyte basophile, 4. Late metamyelocyte basophile, 5. Basophile. MGG stain. 100× objective magnification.

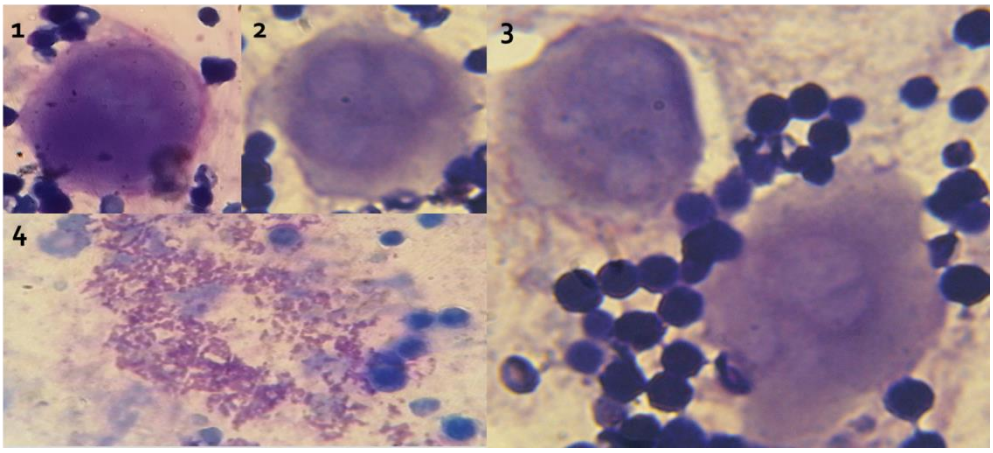


Figure 4.6 megakaryocyte precursors in bone marrow smear. Higher magnification reveals megakaryocyte precursors consisting of different cell types, (1) megakaryoblast, (2) promegakaryocyte, (3) megakaryocyte, and (4) platelets. MGG stain. 40× objective magnification.

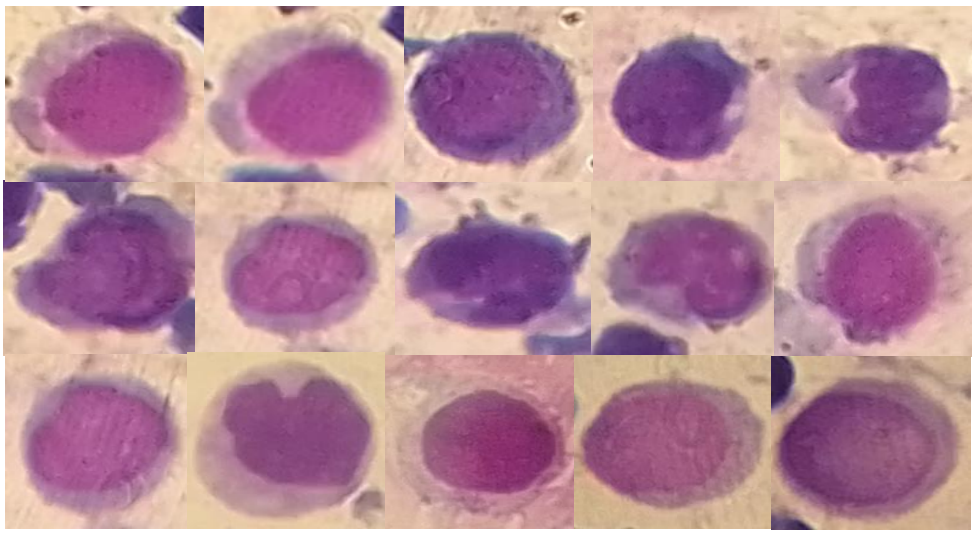


Figure 4.7 Monocyte precursors in bone marrow smear. Higher magnification reveals monocyte precursors consisting of different cell types and shape of monoblast, high N/C ratio with lightly basophilic cytoplasm. MGG stain. 100× objective magnification.

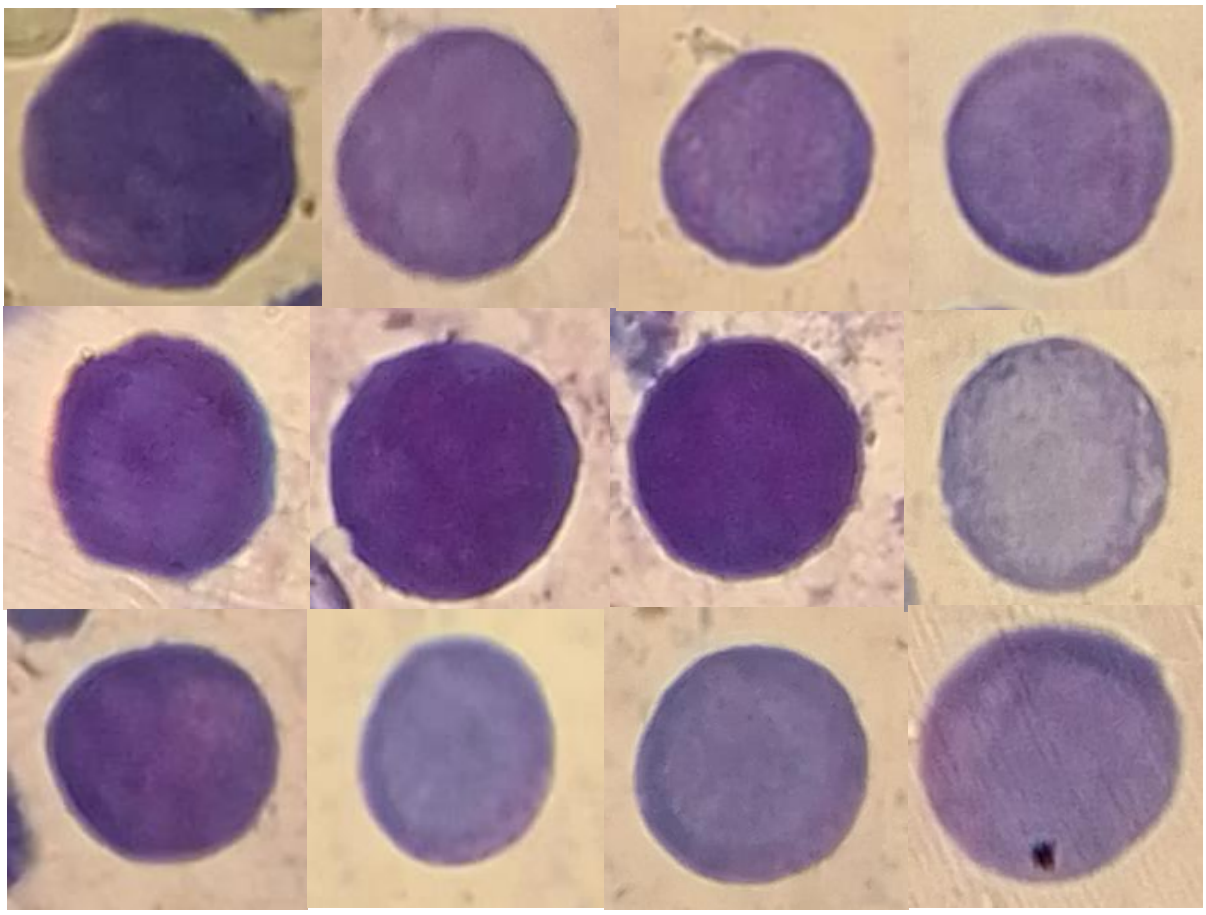


Figure 4.8 Lymphocytes in bone marrow smear. Higher magnification reveals different lymphocytes. MGG stain. 100× objective magnification.

4.1.2 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 identification of hematopoietic cells demonstrated a slight fluctuation in the quantities of derived cells. And that these numbers differed slightly with age compared to the age of one month, as those averages were compared between the ages of the study based on the age of one month as a reference and linking those averages with age. The number of examined

cells and their average numbers were calculated based on cell morphological features documented on the basis of 3 slides/animal/age studied.

The effect of age on erythrocyte precursors revealed no variations in the average number of both rubriblasts and prorubricytes number during the first three-month-old, figures 4.9, and 4.10.On the other hand, both rubricyte and metarubricyte average numbers showed significant variations as age progress with respect to one month old at $P \leq 0.05$, figures 4.11, and 4.12. In addition to that, the average number of reticulocytes exhibited significant elevation in rats' bone marrow smear of three months old compared to one month old, and to two months old at $P \leq 0.05$. Figure 4.13

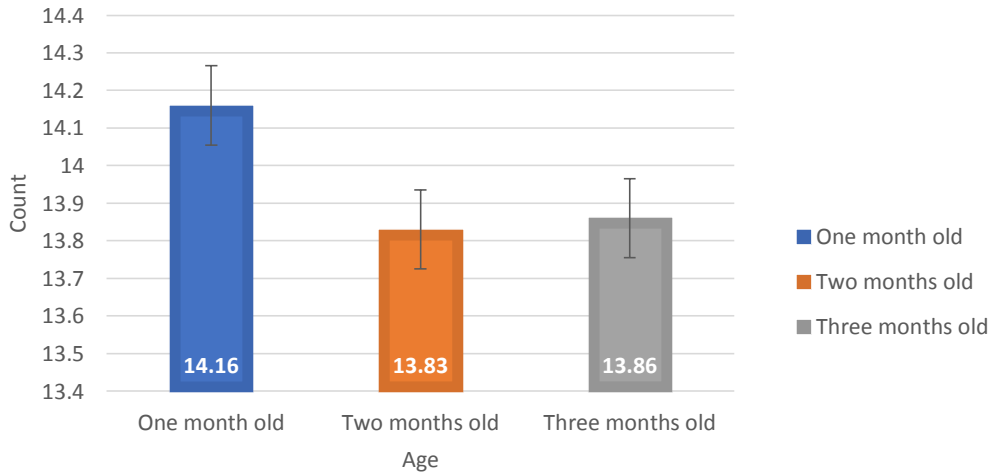


Figure 4.9: The average amount of rubriblasts in healthy rat bone marrow and its relationship to age.

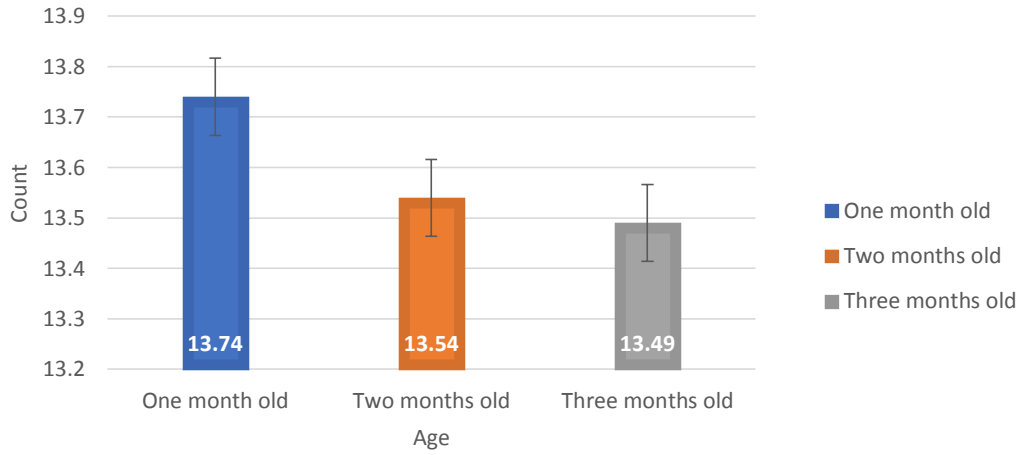
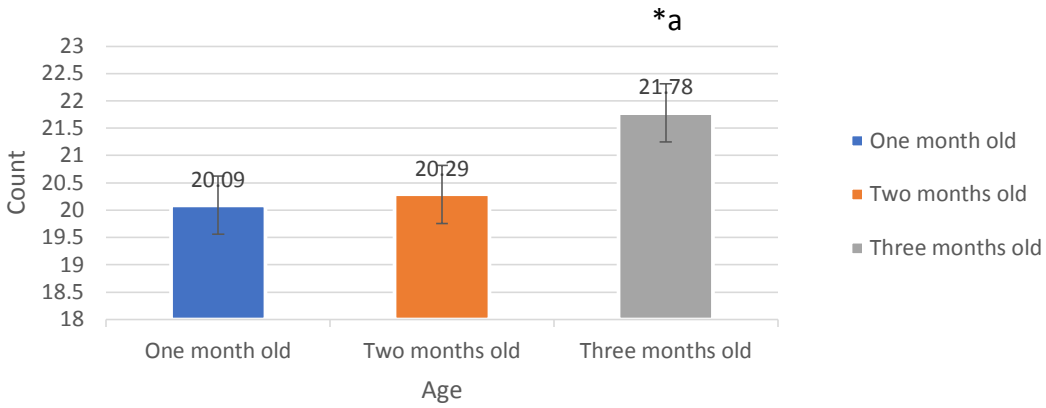
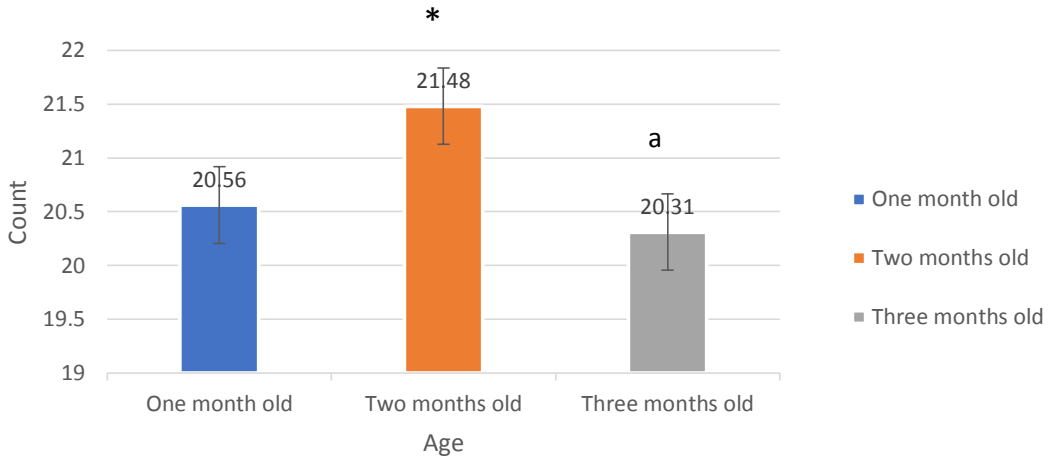


Figure 4.10: Age-related changes in the average prorubricyte count in healthy rat bone marrow



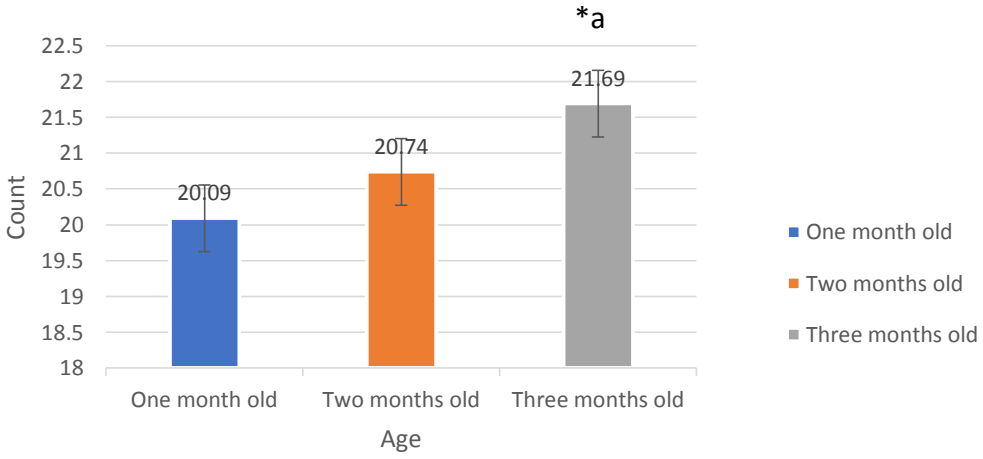
* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Figure 4.11: Average amount of rubricytes in healthy rat bone marrow and their relationship to age



* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

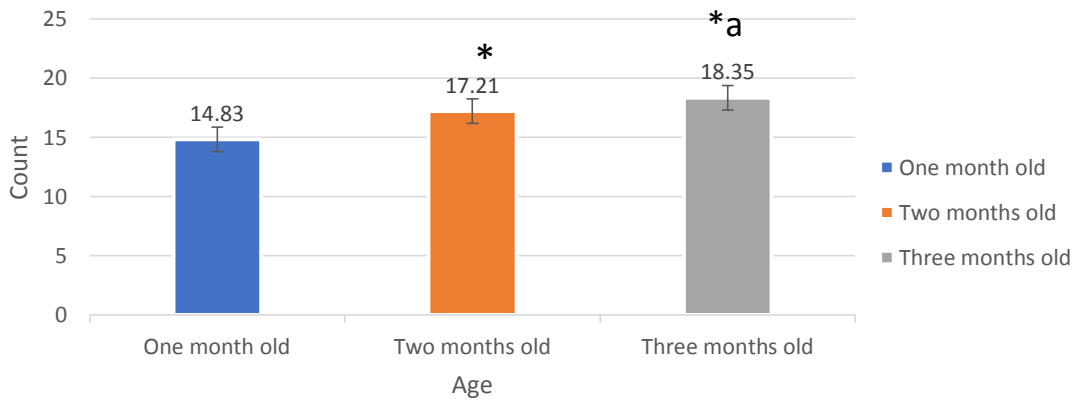
Figure 4.12: Age-related changes in the average number of metarubricytes in normal rat bone marrow



* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

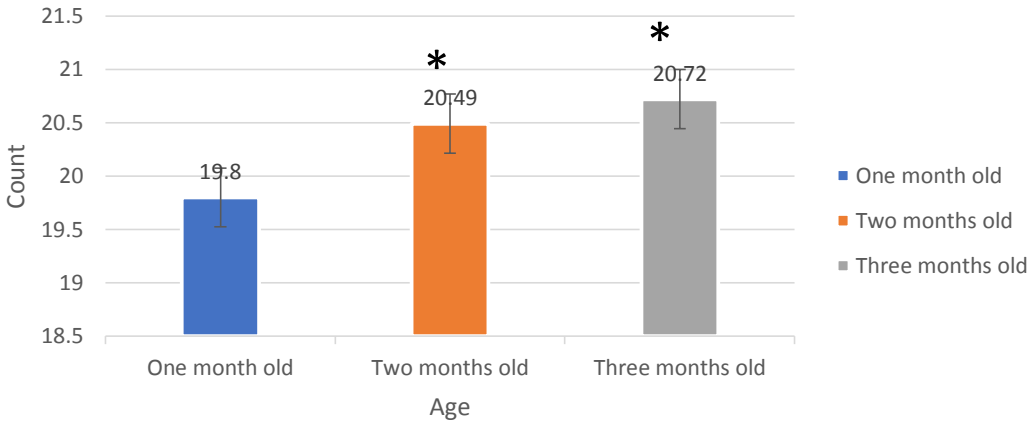
Figure 4.13: The average number of reticulocytes in a healthy rat's bone marrow and their relationship to age

Leukocyte precursors showed significant elevation in the average numbers of all myelocyte precursors as well as differentiated myelocytes starting from myeloblast till band cells, this elevation was clearly noticed in rats' smears of two, and three months old with respect to one month old as a reference for effect of age progress on average myeloid cell count at $P \leq 0.05$. Furthermore, monocyte precursors and monocyte average count exhibited significant decline in their average numbers in rat's smear of two, and three months old. While, lymphoid cells showed non-significant decrease in their average number in comparison between one month old rats and two, three months old at $P \leq 0.05$. Figure 4.14, 4.15, 4.16, 4.17, 4.18, 4.19, 4.20, and 4.21.



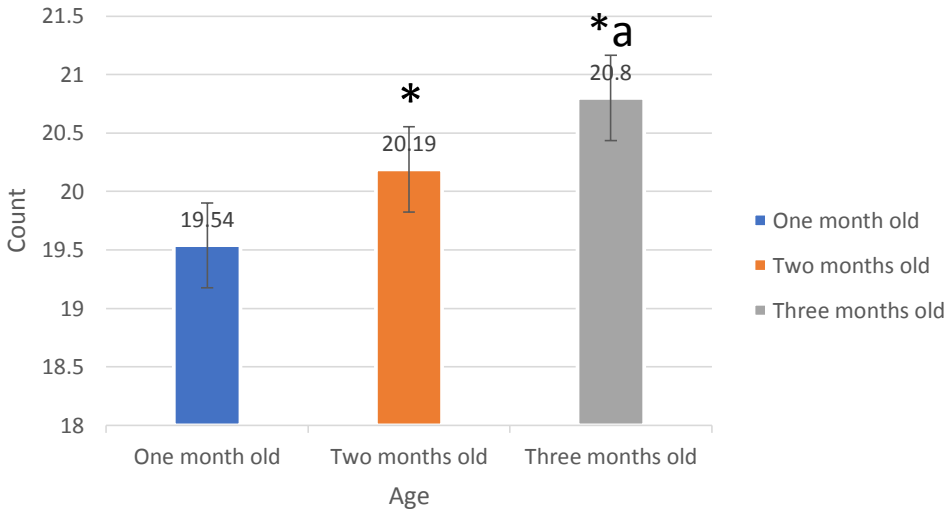
* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Figure 4.14: Age has an impact on the average number of myeloblasts in healthy rat bone marrow.



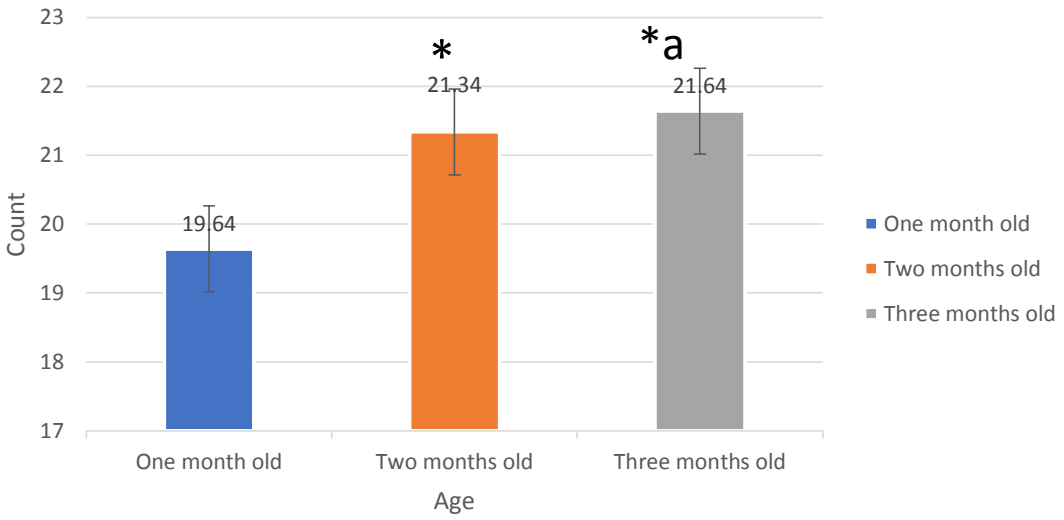
* Means significant at P≤0.05 with one month old,

Figure 4.15: The average amount of promyelocytes in a healthy rat's bone marrow and their relationship to age



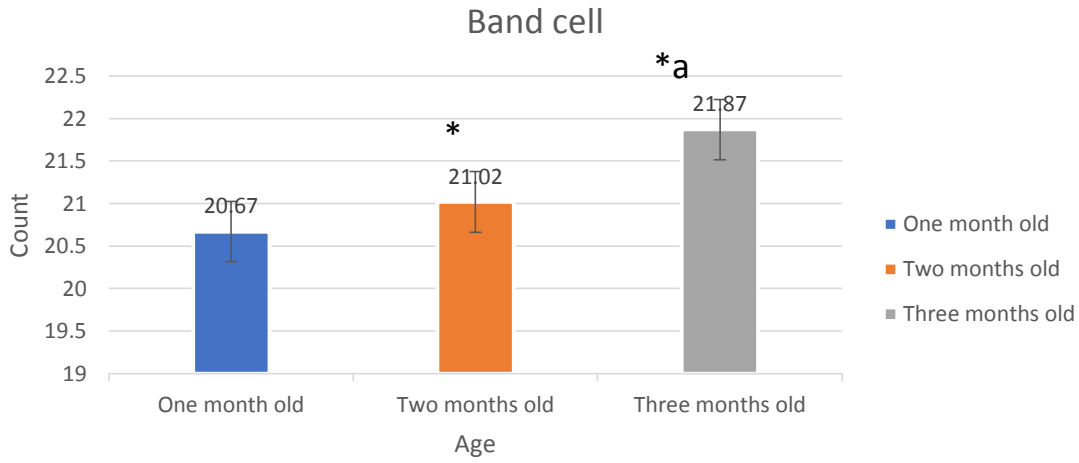
* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Figure 4.16: Age-related variations in the average number of myelocytes in normal rat bone marrow



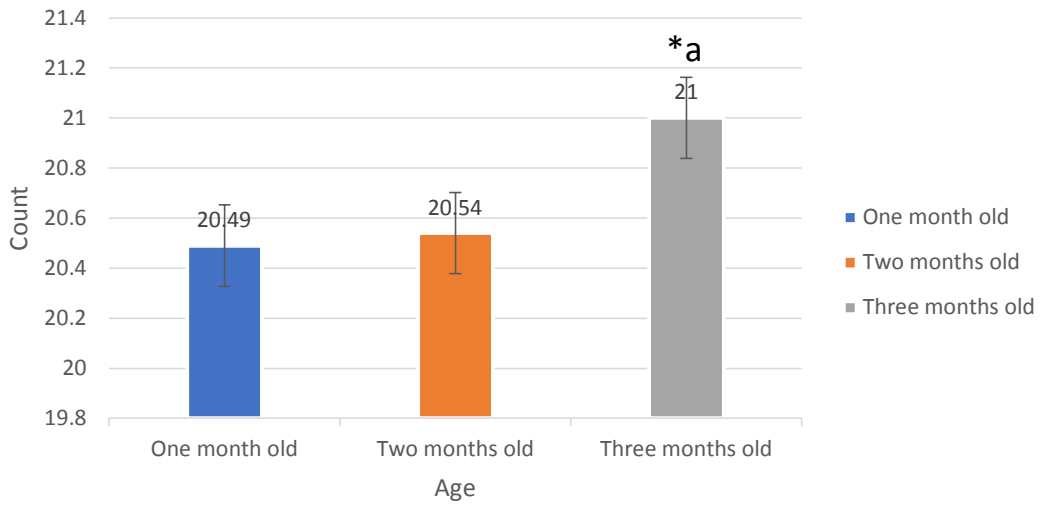
* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Figure 4.17: Age-related variations in the average number of metamyelocytes in normal rat bone marrow



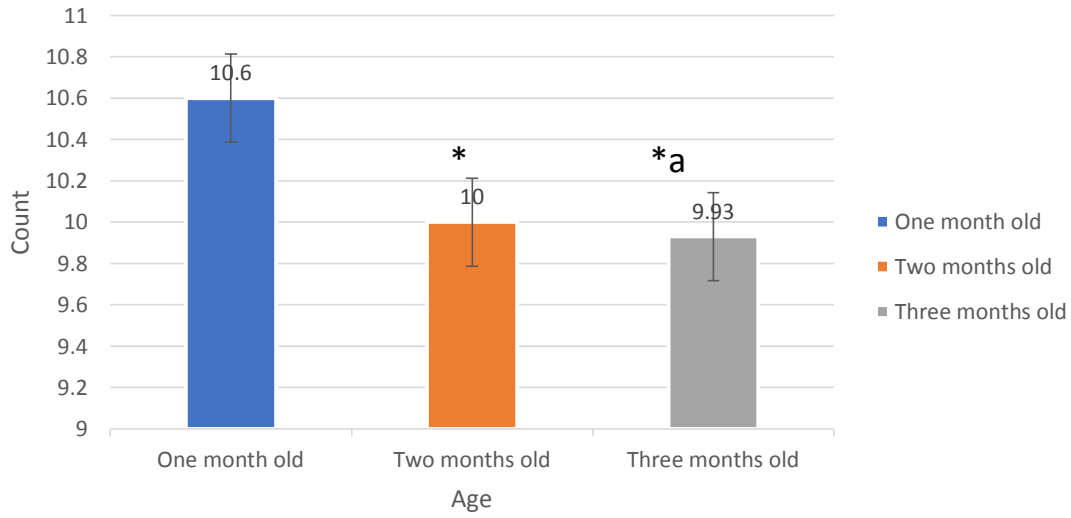
* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Figure 4.18: Age-related alterations in the average number of band cells in rat bone marrow of healthy rats



* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Figure 4.19: Age-related variations in the average segmented granulocyte count in a healthy rat



* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Figure 4.20: Age-related changes in the average amount of monocytes in normal rat bone marrow

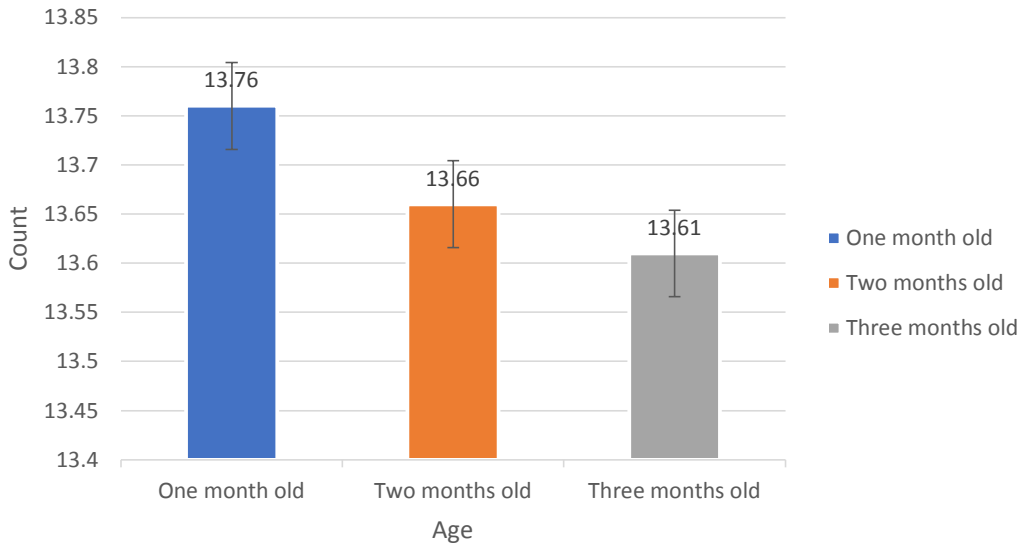


Figure 4.21: Age-related variations in the average lymphoid cell in healthy rat bone marrow

4.2 Gene expression of Hsps and antioxidant enzymes in healthy rat's bone marrow cells.

The analysis of the gene development of multiple heat shock protein and antioxidant enzyme genes demonstrated that the levels of the analyzed genes altered considerably in the bone marrow specimens from healthy rats. Where the ct value and number of fold changes for these genes were used to examine the study's findings, as follows:

4.2.1 Study the gene expression of Hsps in healthy rat's bone marrow

In comparison to the first and second months, which showed balanced upstream regulation of the same gene, Table 4.3 revealed a downstream

regulation of the heat shock gene 90 in bone marrow specimens from 3-month-old rats. Nevertheless, the heat shock protein genes 90 and 27 did not display any appreciable variations during the study period for the same rats.

Table 4.3: effect of age on Hsps genes expression

Age *Hsps*	*One month old*	*Two months old*	*Three months old*	*P-value*
Hsp 90α	19.944 ± 4.51	17.511 ± 2.29	***30.248 ± 4.39*a***	***0.038***
Hsp 90ß	22.174 ± 5.38	33.830 ± 2.58	24.968 ± 3.10	0.066
Hsp 27	31.166 ± 4.98	32.210 ± 4.14	31.628 ± 4.58	0.881

* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Hsp 90α expression revealed increase in fold change up to 24-fold during the age of three months higher one and two months old, figure 4.22.

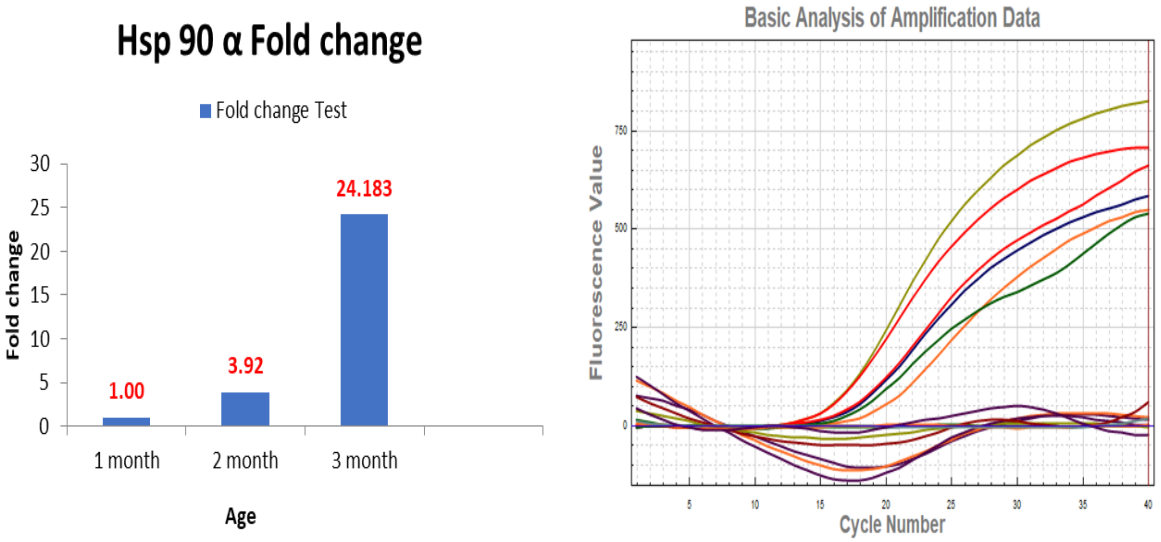


Figure 4.22: Gene expression and fold change of Hsp90α in rat's bone marrow cells

Hsp 90β gene expression showed did not exhibit variation in fold change despite of its upstream regulation during first three months old in healthy rat's bone marrow cells with fold change 9.6, 14.6, and 12.5 respectively, Figure 4.23.

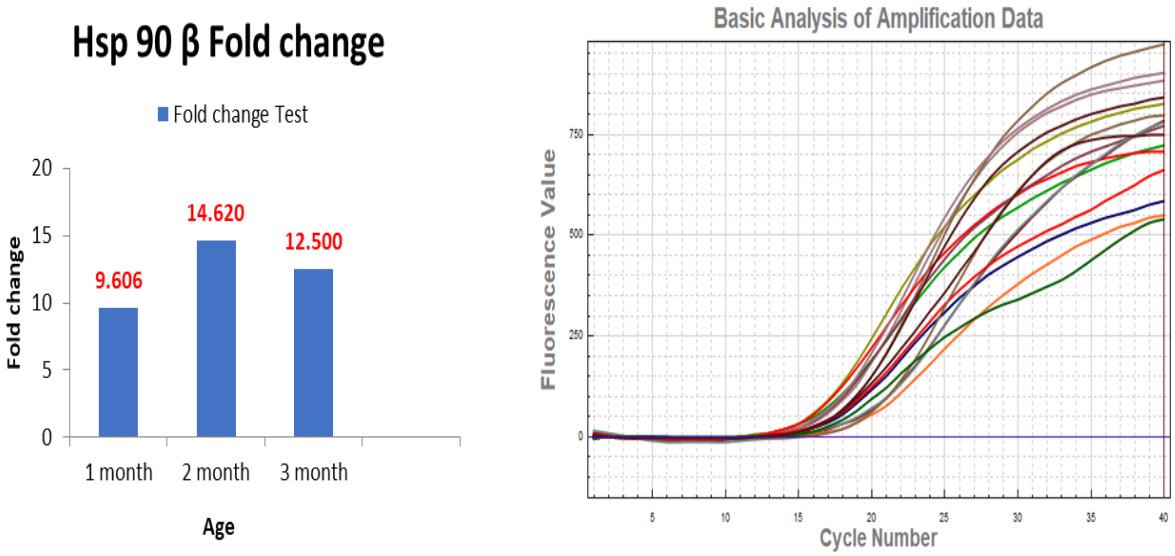


Figure 4.23: Gene expression and fold change of Hsp90β in rat's bone marrow cells

Hsp 27 gene regulation was noticed equally during first three months despite of there was low fold change in two months old of studied rats reach to 0.209 compare to 1st and 3rd month old which vary between 1.590-1.372-fold change with downstream regulation, figure 4.24.

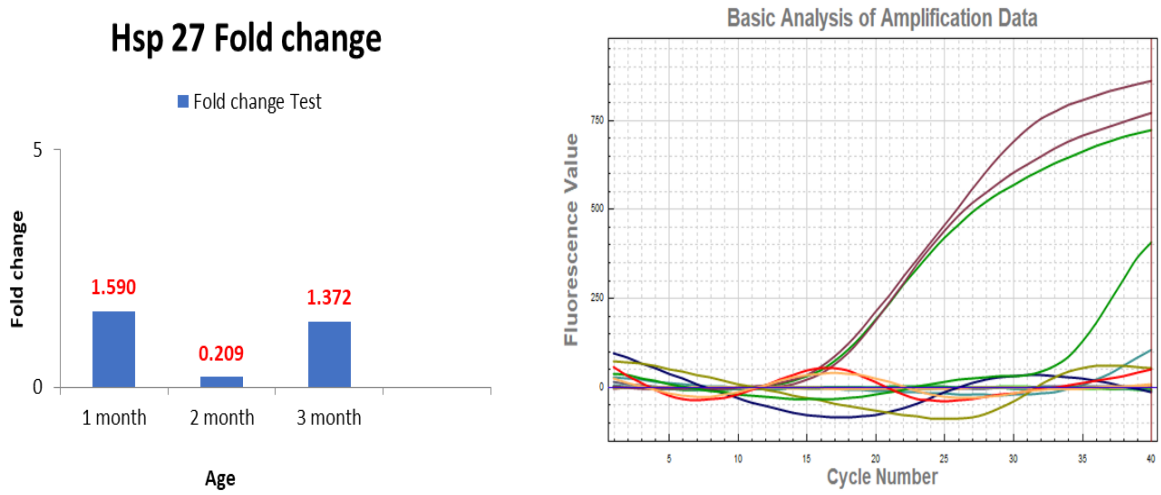


Figure 4.24: Gene expression and fold change of Hsp27 in rat's bone marrow cells

4.2.2 Study the gene expression of antioxidant enzymes in healthy rat's bone marrow

Glutathione peroxidase1 mRNA expression in healthy rat's bone marrow cells showed significant upstream regulation during age of two and three months with respect to one month old at $P \leq 0.05$. While, catalase and superoxide dismutase enzymes expression remain constant with no variation during 1st three months old, Table 4.4.

Table 4.4: effect of age on antioxidant enzymes genes expression

Age ***Hsps***	***One month old***	***Two months old***	***Three months old***	***P-value***
Catalase	20.311 ± 3.21	26.534 ± 4.22	27.742 ± 1.67	0.054
SOD 3	13.340 ± 0.22	13.842 ± 0.62	12.678 ± 0.66	0.145
GPX 1	37.562 ± 0.83	***35.494 ± 0.80 ****	***29.884 ± 0.81 *a***	***0.000***

* Means significant at P≤0.05 with one month old, small litters mean significant differences within groups

Once compared to the number of catalase-fold changes in the bone marrow cells of 1-month-old rats, it was a rise in the number of catalase protein fold changes in the 2-month-old rats' bone marrow samples (746.428), followed by a small but visible decline in the same enzyme's fold changes in the 3-month-old samples (523.483). (326.109), figure 4.25.

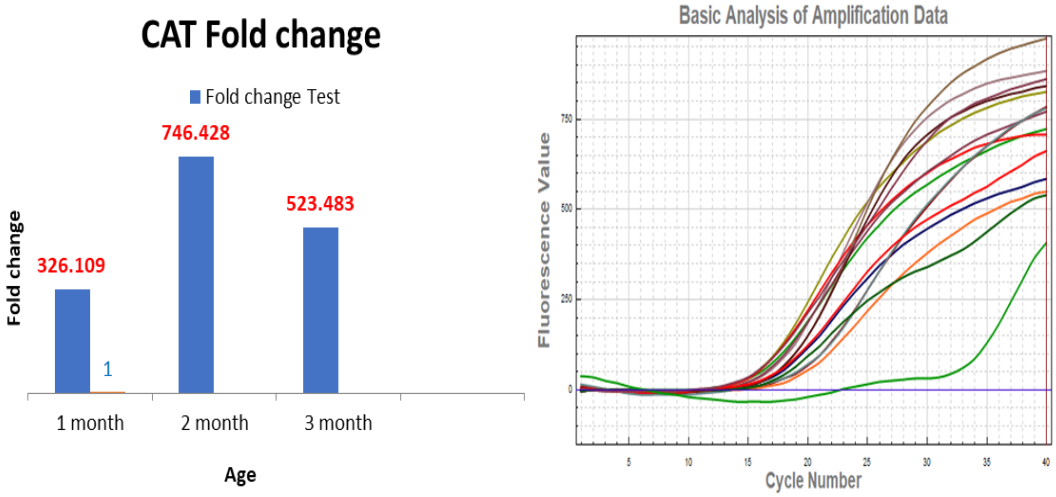


Figure 4.25: Gene expression and fold change of catalase in rat's bone marrow cells

Analysis of the gene expression data of superoxide dismutase showed a twofold increase in the number of its folds change with age in the bone marrow cells of the study rats, as shown in the figure 4.26.

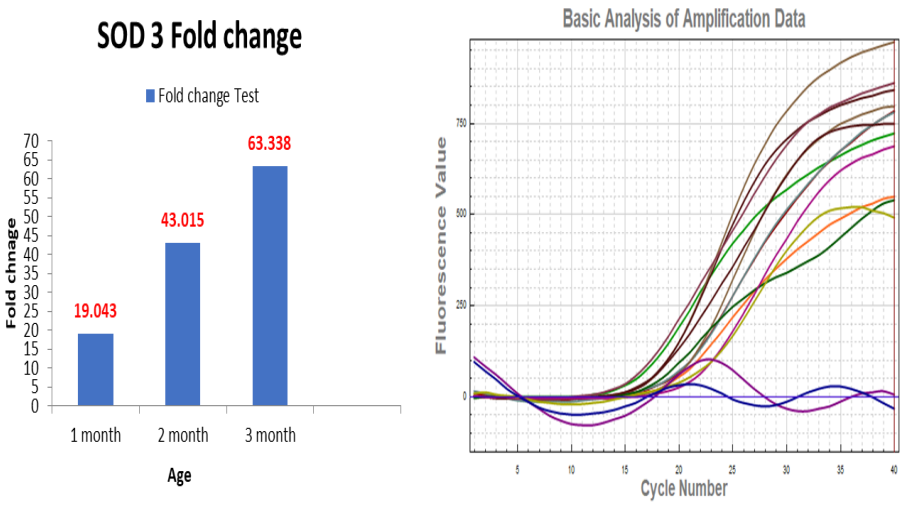


Figure 4.26: Gene expression and fold change of SOD3 in rat's bone marrow cells

In furthermore to the aforementioned, in the bone marrow samples of rats at the ages of 2 and 3 months compared to the first month of life, the glutathione peroxidase 1 enzyme showed a slight change in the number of folds with such a clear rise in the number of its folds despite the decline in these folds compared with the rest of the other antioxidant enzymes, figure 4.27.

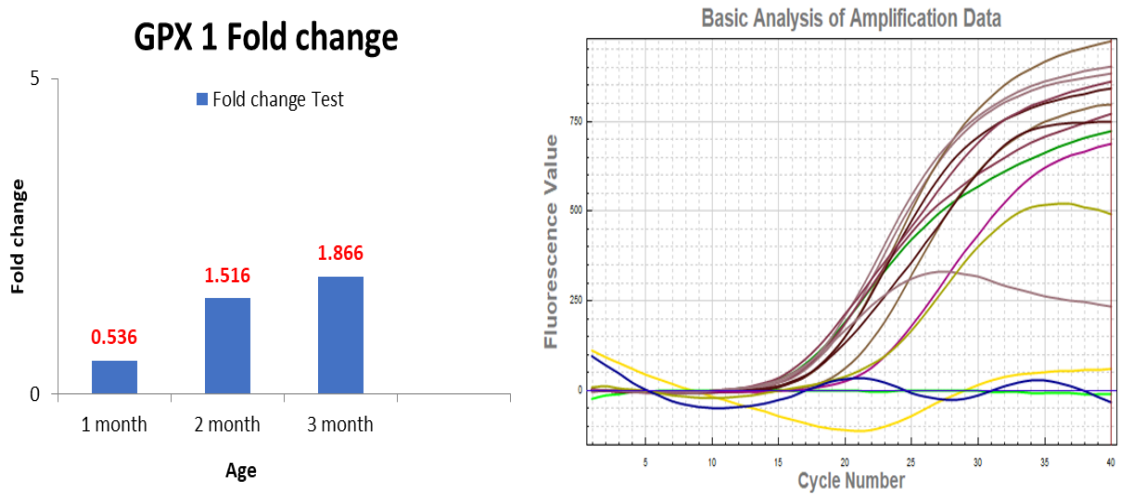


Figure 4.27: Gene expression and fold change of GPX1 in rat's bone marrow cells

4.3 The correlation between Hsps and antioxidants and between gene expression and haematopoietic cells in healthy rat's bone marrow cells

4.3.1 Correlation between Hsps and antioxidants expression in healthy rat's bone marrow cells

The study of the relationship between the studied genes in the healthy rat's bone marrow cells of rats showed a strong inverse relationship at a significant level of more than 0.001 between the gene of heat shock protein 90α and the gene of the antioxidant enzyme glutathione peroxidase1 with a correlation value (r= - 0.357), While superoxide dismutase 3 non-significant inverse relationship (r =- 0.058), and catalase showed also non-significant positive correlation (r = 0.084) with the same Hsp. Moreover, superoxide dismutase 3 revealed strong direct relationship with Hsp 90β at significant level above 0.001 with r value 0.315, meanwhile, other studied antioxidant genes showed non-significant positive direct relationship with Hsp90β too. A slight inverse relationship between Hsp 27 and catalase and superoxide dismutase 3 as well as a slight non-significant direct relationship with glutathione peroxidase 1 as seen in the table 4.5 were noticed, but no significant relationship between the genes of antioxidant enzymes and the heat shock protein 27 gene was encountered.

Table 4.5: Correlation between Hsps and antioxidants gene expression in healthy rats' bone marrow						
Antioxidants Hsps	***Catalase***		***SOD3***		***GPX 1***	
	r-value	***P-value***	***r-value***	***P-value***	***r-value***	***P-value***
Hsp 90α	0.084	0.231	-0.058	0.411	-0.357**	0.000
Hsp 90ß	0.117	0.096	0.315**	0.000	0.080	0.253
Hsp 27	-0.060	0.393	-0.065	0.352	0.023	0.739

**. Correlation is significant at the 0.01 level.

4.3.2 The correlation between progenitor cells in healthy rat bone marrow and gene expression profiling

Rats with healthy bone marrow showed a strong inverse relationship between all erythroid cell precursors and Hsp 90, excluding reticulocytes, and a strong direct relationship with Hsp 27, is from an analysis of the relationship between heat shock proteins and the quantity of erythrocyte precursors. Hsp 90 expression displayed an inverse relation with rubricyte average number, while Hsp 27 expression demonstrated an opposite relation with reticulocyte number .Table 4.6.

Table 4.6: Correlation between Hsps and number of erythrocyte precursors in healthy rats' bone marrow						
Hsps / ***Erythroid cells***	***Hsp 90 α***		***Hsp 90 β***		***Hsp 27***	
	r-value	***P-value***	***r-value***	***P-value***	***r-value***	***P-value***
Rubriblast	-0.097	0.167	-0.232**	0.001	0.189**	0.007
Prorubricyte	-0.044	0.527	-0.416**	0.000	0.276**	0.000
Rubricyte	-0.223**	0.001	-0.099	0.158	0.155*	0.027
Metarubricyte	0.003	0.965	-0.591**	0.000	0.131	0.062
Reticulocyte	0.072	0.302	0.081	0.249	-0.306**	0.000

**. Correlation is significant at the 0.01 level.

*. Correlation is significant at the 0.05 level.

The numbers of rubriblasts and prorubricytes have a strong direct correlation with catalase, as per statistical research on the interaction between antioxidant enzymes and haematopoietic cells (r = 0.343, 0.152) and superoxide dismutase (r = 0.304, 0.162) respectively, and inverse relationship between super oxide dismutase and rubricyte, metarubricyte (r = -0.207, -0.174). Furthermore, glutathione peroxidase1 showed inverse relationship with prorubricyte and direct relationship with rubricyte numbers (r = -0.158, 0.297) respectively. Table 4.7

Table 4.7: Correlation between antioxidant genes and number of erythrocyte precursors in healthy rats' bone marrow						
Hsps / ***Erythroid cells***	***Catalase***		***SOD3***		***GPX 1***	
	r-value	***P-value***	***r-value***	***P-value***	***r-value***	***P-value***
Rubriblast	0.343**	0.000	0.304**	0.000	-0.115	0.099
Prorubricyte	0.152*	0.030	0.162*	0.020	-0.158*	0.024
Rubricyte	-0.068	0.332	-0.207**	0.003	0.297**	0.000
Metarubricyte	-0.120	0.086	-0.174*	0.013	0.131	0.061
Reticulocyte	-0.130	0.064	-0.127	0.069	0.062	0.374

**. Correlation is significant at the 0.01 level.

*. Correlation is significant at the 0.05 level.

The relationship between the Hsp 90α, Hsp 27 and all types of granulocytes progenitors and immature granulocytes were strong inverse relationship, meanwhile, Hsp 90β exhibited strong inverse relationship with myeloblasts and promyelocytes. Furthermore, Hsp 90β exhibited strong direct relationship differentiated myelocytes and their immature cells. Table 4.8.

Table 4.8: correlation between Hsps and number of granulocyte precursors in healthy rats' bone marrow						
Hsps / ***Erythroid cells***	***Hsp 90 α***		***Hsp 90 β***		***Hsp 27***	
	r-value	***P-value***	***r-value***	***P-value***	***r-value***	***P-value***
Myeloblast	-0.297**	0.000	-0.181**	0.010	-0.108	0.125
Promyelocyte	-0.034	0.625	-0.180**	0.010	-0.059	0.404
Myelocyte	-0.226**	0.001	0.358**	0.000	-0.354**	0.000
Metamyelocyte	-0.085	0.228	0.367**	0.000	-0.029	0.675
Band cell	-0.287**	0.000	0.297**	0.000	-0.254**	0.000
Segmented granulocytes	-0.303**	0.000	0.263**	0.000	-0.228**	0.001

**. Correlation is significant at the 0.01 level.

It is noteworthy that, during the study period, all antioxidants had a strong positive direct interaction with all leukocyte progenitor cells in the healthy bone marrow of rats, with the exception of glutathione peroxidase1, which had a slight inverse relation with promyelocytes (r = -0.151). Table 4.9.

Table 4.9: correlation between antioxidant genes and number of granulocyte precursors in healthy rats' bone marrow

Hsps / Erythroid cells	*Catalase*		*SOD3*		*GPX 1*	
	r-value	*P-value*	*r-value*	*P-value*	*r-value*	*P-value*
Myeloblast	0.321**	0.000	0.335**	0.000	0.511**	0.000
Promyelocyte	0.290**	0.000	0.378**	0.000	-0.151*	0.031
Myelocyte	0.110	0.118	0.129	0.065	-0.070	0.322
Metamyelocyte	0.173*	0.013	0.138*	0.048	-0.048	0.495
Band cell	0.159	0.315	0.405**	0.001	0.449**	0.000
Segmented granulocytes	0.037	0.601	0.301**	0.000	0.435**	0.000

**. Correlation is significant at the 0.01 level.

Monocyte precursors showed strong inverse relationship with the two types of Hsp90 as well as catalase and superoxide dismutase, while showed non-significant inverse relationship with lymphoid cells. Furthermore, glutathione peroxidase exhibited strong direct relationship with both monocyte and lymphoid precursors. Also, Hsp 27 exhibited non-significant direct correlation with lymphoid and monocyte precursors after data analysed by using Person correlation test at $P \leq 0.05$. Tables 4.10, 4.11.

Table 4.10: Correlation between Hsps and number of agranulocyte precursors in healthy rats' bone marrow						
Hsps / Agranulocyte precursors	*Hsp 90 α*		*Hsp 90 β*		*Hsp 27*	
	r-value	*P-value*	*r-value*	*P-value*	*r-value*	*P-value*
Monocyte precursor	-0.314**	0.000	-0.412**	0.000	0.105	0.135
Lymphoid cells	-0.080	0.257	-0.048	0.493	0.107	0.127

**. Correlation is significant at the 0.01 level.

Table 4.11: Correlation between antioxidant gene and number of agranulocyte precursors in healthy rats' bone marrow						
Hsps / Agranulocyte precursors	*Catalase*		*SOD 3*		*GPX 1*	
	r-value	*P-value*	*r-value*	*P-value*	*r-value*	*P-value*
Monocyte precursor	-0.148*	0.034	-0.009	0.901	0.344**	0.000
Lymphoid cells	-0.136	0.051	-0.054	0.442	0.640**	0.000

**. Correlation is significant at the 0.01 level.

Chapter five

The discussion

In order to sustain healthy hematopoiesis and a quiescent state with the desired rate of self-renewal, the bone marrow stem and self-renewal cells' activity is often tied to their bone marrow microenvironment. The current study's main purpose was to evaluate the relationship between haematopoiesis and age, to profile the gene expression of specific Hsps and antioxidant enzymes in the bone marrow of healthy rats, and to determine the linkage between Hsps, antioxidants, and studied gene profiling and haematopoiesis.

The results were exhibited no variations noticed in the 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 precursors 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. Multipotent stem cells are a class of heterogeneous haematopoietic stem cells that can self-renew and differentiate into several types of the body's productive blood cells (Asumda and Chase, 2011). Parmar *et al*., 2007, demonstrated that the BM seems to be where developed mammalian haematopoiesis originates. Here, a small number of quiescent stem cells give rise to a lot number of committed progenitors. Our findings are in consistent with (Parmar *et al.*, 2007) who illustrate how committed cells replenish all blood cell lineages over the lifespan of an organism. Also, the proliferative possible of hematopoietic stem cells (HSCs) is thus

appreciable being enriched with the special ability to bolster themselves by self-renewal (Szade *et al.*, 2018).

The marked elevation in number of myeloid cells with age could be come as a result of body requirements for these multilineage haematopoietic cells and their role in body defense mechanisms including immune system. The low average number 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 additionally as a result of their capacity for self-renewal and specific degree restriction of multipotency to develop into different cell types. This opinion is consistent with what the other researchers pointed out (Weissman, Anderson and Gage, 2001), 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 capability of these cells to form two vital lineages of essential cells in the cellular formation of the bone marrow is just what provides them their value. Owing to its high potential to differentiate into mainly two lineages of bone marrow cells, such as hematopoietic and mesenchymal, these cells are significant. However, because of their characterized pluripotency, these cells may occasionally undergo trans-differentiation and produce irrelevant cell types (Zhang *et al.*, 2003). Furthermore, because of the progenitor cells have the ability to roam and diffuse into other tissues (Belyavsky *et al.*, 2021) These are vital roles in determining how they are distributed inside the bone marrow tissue.

The results of the study indicate that age, which affects many intrinsic characteristics of HSCs by influencing on their capacity, performs another

role of maintaining self-renewal and differentiation capacity over time, independent of their microenvironment (Mejia-Ramirez and Florian, 2020). The findings of this study concur with those of (125), who demonstrated that the frequency of human bone marrow hematopoietic stem cells rises with age and becomes more myeloid-biased due to higher in myeloid differentiation potential and a decrease in lymphoid differentiation potential. 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. Yamamoto et al., 2018, illustrates that the quantity of multipotent HSCs does not rise significantly with age, but the myeloid-restricted repopulating progenitors subpopulation does (Yamamoto *et al.*, 2018). Interestingly, the outcomes of the study concur with those of (Sudo *et al.*, 2000), who observed that mice's HSCs demonstrate a clear drop in lymphopoiesis and an increase in myelopoiesis throughout age. In general, increase in myelopoiesis, and decrease in lymphopoiesis during aging could be affected by different cellular pathways within the niche as an interaction between these cells and their aging niche, for instance, there is evidence that the HSC populations respond differently to cytokines like IL-7 and TGF- in young and old mice (Challen *et al.*, 2010). 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 quite supported by (Cho, Sieburg and Muller-Sieburg, 2008; Beerman *et al.*, 2010), In early, 2-month-old mice, just a tiny portion of myeloid-partisan cells may be identified with

balanced-potential HSC, demonstrating that the early mouse HSC pool may comprise clones that are lineage-biased and compete for niche 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. A stability between free radicals and antioxidants, both enzymatic and non-enzymatic, is one of the noticeable and well-known facts of a case of oxidative stress. This will alleviate the harmful impacts of these radicals and cease or alleviate their attack on proteins, fats, and nucleic acids in cells, which provokes unwanted effects at the cellular level (Picou *et al.*, 2019).

The goal of the ongoing research is to understand the gene expression of a few Hsps genes, which respond as a cellular chaperon to avert any transformation of the nature of cellular proteins dictating the role and differentiation of HSCs in the bone marrow, as well as a few genes that manage cellular antioxidant enzymes. These antioxidants notably assist in maintaining the microenvironment of bone marrow cells, which is critical to the development and differentiation of HSCs (He *et al.*, 2017). The gene expression of various Hsps was very evident in the bone marrow cells in the present study, which enables us to understand how well these understanding of the process in the niche-mediated regulation of the equilibrium between the activation and latency of various progenitors in the bone marrow. Hsp90α and 90β exhibited an increase in fold change with in the age, this could be due to the fact the largest number of proteins should fold into

particular structures to meet their activity and function and within the niche, freshly synthesized proteins could be at risk of misfolding and forming unwanted proteins. This is in agreement with (Balchin *et al.*, 2016) who explain how cellular chaperones to receive the newly protein chain from ribosome and mentor it along a productive folding path to ensure efficient folding. On other hand, as protein is structurally dynamic and constant by network of chaperones is necessary for keeping protein homeostasis which known as proteostasis (Noormohammadi *et al.*, 2017). This endorses the study findings that the capacity of this proteostasis declines with aging due to protein aggregation, that further provokes pathological conditions, and that the number of folds of heat shock proteins, which plays a vital and regulatory role in the environment of bone marrow cells. In general, the bone marrow niche works with certain range of stress as consequence to hypoxia which is very necessary to haematopoiesis (Wielockx *et al.*, 2019). In order to protect proteins from damage and maintain proteostasis, the cell's chaperones operate to prevent the creation and ejection of free radicals, which would otherwise attack and alter proteins and change their nature. This unfolded protein response takes place in the endoplasmic reticulum (Noormohammadi *et al.*, 2017). Another explanation for increased protein folds is linked with protein chaperones function within the cell by helping the correct folding proteins to perform their normal function within the HSC or progenitor cells.

Conversely, the integrity and self-regeneration of stem cells are directed by cellular factors such as transcription factors in accordance with specific HSPs (Niwa *et al.*, 1998). The current finding is in agreement with (Gao *et al.*, 2014) He stated that HSP90 is a fundamental and crucial protein for self-renewing stem cells because it links to one of the internal factors

(STAT3/Hop) by activating and modifying this factor. Therefore, by minimizing the negative impact caused by internal and external stress elements within the bone marrow niche, chaperone expression is beneficial in stopping the stemness of different SCs. Likewise, our findings are in agreement with (Baharvand *et al.*, 2007), which express that upregulation levels of Hsps and co-factors in SCs can apply a buffering response contra external and internal stressors, via lasting their stemness. Also, (Baharvand *et al.*, 2008) supported the research findings by their research findings via their observation of a down-steam gene expression in SCs differentiation that leads to restricting gene expression heat shock proteins are greater than gene expression of new genes and these changes in heat shock proteins play a key role in cell differentiation as biomarkers specific to stem cells. This is very meaningful to sign that downstream of Hsps may happen as downregulation expression earlier to noticeable expression of traditional differentiation markers. In current findings, Hsp27 expression showed inverse relationship with myeloid cell differentiation via its downstream expression in healthy bone marrow cells. This result was in agreement with the result of (García-Bermejo *et al.*, 1995) who explored the connection between Hsp27 and Hsp60 and myeloid commitment by intervening as general sparks for essential monocyte-macrophage receptors, causing upregulation of these cells Hsps primarily support many intracellular processes and signals, such as facilitating native protein folding, maintaining multiprotein complexes, intraorganellar protein shuttling, and developing protein deterioration. Moreover, playing a critical role in HSCs via their interaction with transcript factors such as STAT3, Oct4 and signaling pathways, which may inflect HSCs differentiation and proliferation and This

viewpoint is compatible with the results from a previous study on the role of heat shock proteins in stem cell behavior (Fan, 2012).

In agreement with our results (Signer *et al.*, 2014) who unearthed that adult HSCs have a lesser protein synthesis rate and protein folding storage than other SCs, which likely reflects the cell cycle status of silent HSCs HSCs enhance protein synthesis during prenatal life and in proliferative states. Because of the scarcity of protein folding, HSCs contribute to the accumulation of un-/misfolded proteins and the subsequent induction of the unfolded protein response (UPR), primarily the endoplasmic reticulum (ER) stress response (Sigurdsson *et al.*, 2016). When oxidative stress appears at a certain level in the stem cell, it induces several cellular responses, including cell cycle arrest and apoptosis, and this is a severe defense program in the stem cell to prevent the accumulation of un/mis-folded proteins, which is thought to lead to tumor shift. The current findings revealed that there are increase in protein fold change in certain studied genes during the study. This result could be due to protein synthesis, in this process, the peptides are twisted into folds, with supported from glycosylation and molecular chaperones. For instance, of these molecular chaperones Hsps, protein disulfide isomerases and prefoldin complexes. Evidently, in certain cellular stressful conditions, such as oxidative stress, hypoxia, and endoplasmic reticulum overcapacity, the folding system can be deficient, tends to result in the induction of un-folded and mis-folded proteins, and this opinion is consistent with (Englander and Mayne, 2014). Besides, the identification of such unconventional proteins by cellular chaperones (Hsps) as a consequence of overcapacity of protein folding and deterioration results in a buildup of folded change proteins, which involves the activation of three distinct ER stress response pathways based on the intensity of the stress,

triggering multiple biological reactions. Also, this opinion is supported by (Sigurdsson and Miharada, 2018) whose demonstrates the three ER stress response pathways related to severity of cellular stress, these pathways are non-stressed condition, modest/transient stress, and robust/chronic stress that leading to induce fold change proteins under regulation of certain cellular signals mainly regulated survival signals by enhancing protein folding and degrading un-/mis-folded proteins. Within certain conditions, newly synthesized un/miss folded protein that is physiologically and structurally pointless proteins that is vital for the relevant folding of newly synthesized polypeptide chains and unstable protein conformers is produced (Dahiya and Buchner, 2019).

Hsp90 is one of the most abundant and well-regulated cytosolic proteins that are involved in HSCs in two forms: transcriptionally expressed Hsp90β and stress-induced Hsp90α. Hsp90, which frequently appears as homodimers, has the potential to only intervene in the folding and development of transcription controllers and signal transducers; intuitively, it has been mapped with approximately 300 client proteins. (Sreedhar *et al.*, 2004). The most important things that the results of the study reached about the illiteracy of heat shock protein 90 is its relationship to blood progenitor cells, which depends on the type of cells and on the micro-environmental conditions surrounding the progenitor cells. And this result reached to an important point in role of Hsp90 in regulation of differentiation and proliferation of different HSCs, progenitors and immature haematopoietic cells. This opinion supported by (Taipale *et al.*, 2012) It has progressed to the point where Hsp90 is the most complex of all molecular chaperones, with its functional cycle aided by a large cohort of co-chaperones in its chaperone cycle. The indelible concern regarding Hsp90 that split it from

other chaperones is that it is appropriate additionally under unstressed conditions and show in sums seemingly in abundance to typical needs inside the normal functioning of cells. Hsp90 has a very specific set of client proteins for their final maturation or activation in cells, and many of these client proteins are ligand authoritative proteins, so interaction with Hsp90 may stabilize or increase their folding competence, which supports the study findings. It is still unconfirmed whether Hsp90 successfully promotes a conformational change in its clients. (Geller *et al.*, 2018). The finest roles of Hsp are either in chaperone-mediated protein folding, and ongoing findings suggest definite protein folding in different proteins such as enzymatic antioxidants and different Hsps, which is consistent with previous studies of (Fink, 1999; Chen and Inouye, 2008) which showed distinct Hsps have been encountered to respond as chaperones by intervening in intramolecular instinctive events such as folding and intermolecular instinctive events such as intracellular signaling and protein degradation/refolding. A few Hsp work as co-chaperones and are included within the control and tweak of chaperone action. Stem cell self-renewal undergoes many processes under regulation of intrinsic and extrinsic cellular signals in an organized and complex manner for the purpose of regulating their differentiation, the most important of these cellular signals are the molecular chaperones and co-chaperones present within the stem cell itself in its microenvironmental setting. The most important of these cellular chaperones are Hsps, which play a key role in responding to different levels of cellular stress in stem cells and their biosphere for the purpose of differentiation (Duffy *et al.*, 2012; Shende *et al.*, 2019).

Self-renewal, expansion, sustenance, relocation, development, and mobilization of bone marrow (BM)-derived stem and progenitor cells are

influenced by fascinating cell intrinsic/extrinsic signals provided by their microenvironment (Ushio-Fukai and Rehman, 2014). Hence the nature of requirement of niche to keep HSCs differentiation and proliferation to certain level of oxidative stress, current findings showed also an increase in antioxidant enzymes fold change which is protein in nature linked with age. The antioxidant enzyme's role in the body is to scavenge the excess free radicals which are harmful to many cellular proteins and consequence to this action large number of unfolded proteins produce of heat shock response. It is obvious that expanding the gene expression of target genes governing the formation and activity of antioxidant enzymes is strongly linked to the cell signaling of bone marrow cells, where it differs widely under specific conditions of cellular stress caused by the formation of active oxygen species. Fact, HSCs and progenitors participate in various physiologic and pathologic reactions under the control of ROS, particularly H_2O_2. What's more, a low level of H2O2 in steady - state HSCs is critical for their stemness, whereas a high level of H2O2 within HSCs or their specialty promotes HSC or stem/progenitor cell differentiation, expansion, migration, and survival. Excess ROS production is required for physiological cellular functions, whereas excess ROS add value to pathological conditions like as aging or programmed cell death, tends to result in a premature debility of self-renewal in these cells via p38 MAPK activation. This opinion is in agreement with (Sardina *et al.*, 2012). The vast ROS production is very destructive to cells and can lead to a variety of bad outcomes such as aging, diabetes, and cancer. But nevertheless, in accordance with this viewpoint, these ROS at normal levels serve as cellular signaling molecules to perform normal cell functions such as cell proliferation, migration, survival, differentiation, and gene expression (Tauffenberger and Magistretti, 2021).

Overall, H_2O_2 is almost steady and may not react with other free radicals; its been predicted that it will function as a second messenger in physiological redox signaling, and the ROS generation system and antioxidant enzymes are the principal regulatory systems in controlling excessive cellular ROS levels via the adjusting cellular activity system to ensure normal cell function. Throughout normal hematopoiesis, H_2O_2 has cell-specific influence on HSCs and hematopoietic progenitor cells. H_2O_2 's cell-intrinsic effects on stem and progenitor cell function under stress conditions have also been approved by (Dernbach *et al.*, 2004) as well as endorsed our insights, which explored that ROS rates are lower in SCs than in mature HSCs, resulting in higher transcription of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase, and is required for sustaining "stemness" such as an undifferentiated, self-renewing state under oxidant stress.

Our findings revealed relative gene expression of different antioxidant enzymes and this could be due to formation of intracellular ROS which in turn promote antioxidant via activation of their coded genes. This is in agreement with (Maher and Yamamoto, 2010) It was discovered that intracellular ROS at various levels plays a vital regulatory role by controlling oxidative stress, which must be fought back through antioxidative mechanisms, including the upregulation of genetic markers whose expression is regulated by antioxidant response elements. Also, (Allen and Tresini, 2000) endorse our outcomes that varying levels of oxidative stress play a key regulator of gene expression via direct/indirect signaling pathways, or even by adjusting the behavior of certain transcription factors. Moreover, antioxidant gene expression during haematopoiesis in healthy rats was noticed in current study, this might be

due to antioxidant response element (ARE), that play critical role in mediating expression and production of antioxidant enzymes in critical pathway to the cells against overexpressed stress. This viewpoint is reliable with (Jaiswal, 2004), who suggested that the pathway of antioxidant activity against cellular stress is linked by antioxidant response element (ARE) via gene expression and antioxidant enzyme production via nuclear erythroid factor 1 and 2 (Nrf1 and Nrf2), which bind to ARE and enforce ARE-mediated gene expression and production. Even so, Nrf2 does have a massive impact than Nrf1 in binding to ARE, giving rise in ARE-regulated gene expression (Denicola *et al.*, 2011). Further to that, (Tonelli, Chio and Tuveson, 2018), discovered that Nrf2 translocates to the nucleus and attach to the ARE, arising in the organized activation of ARE-controlled genes, and other nuclear factors such as Mafk and cMaf, that either adversely enforce ARE-mediated gene expression. Overall, the process of folding ARE to Nrf2 is figured to categorize the expression of antioxidant enzymes to retain cellular defenses active and/or to cause immediate induced enzymes to normal levels.

The study was carried out on healthy, normal, disease-free rats' elderly 1 to 3 months to understand about the relations between various genes that play key role in the regulation of cellular stress in bone marrow cells, as well as the relationship between those genes and the type of haematopoietic cells based on the nature of the internal environment of the bone marrow and its impact on gene expression under normal conditions for the formation of these cells. The findings of this study support the relationship between the gene expression of the study's various genes and the number and type of hematopoietic cells (long-term self-renewing HSCs, short-term self-renewing HSCs, multipotent progenitors) were very impressive, as it showed

different types of this relationship, some of which have a strong direct relationship with one type of cells and another with another type of cells. For illustration, there is indeed a powerful indirect proportion between the heat shock protein 90 and red blood cell precursors. At the same time, the heat shock protein 90β showed an inverse relationship with myeloblasts and promyelocytes which are differentiated cells, and with the number and type of the leukocyte progenitors showed a direct relationship with the of them during the stages of development and maturation. As well as other studied Hsps showed in same manner of relationship in addition to studied antioxidants enzyme gene expression; i.e., a heterogeneous relationship according to the type of cells in the same way and at the same time, which is attributed to the close relationship between the activity of progenitor cells, their differentiation, development and maturation with the nature of the micro-environment that organizes these cells. As previously stated, the delineation of stem and progenitor cells arises in a balanced state of stress and anti-stress due to the activity of ROS produced by cellular processes in these cells on the one hand, and in the environmental surroundings of these cells due to the influence of other bone marrow cells on the other. This opinion is fully consistent with (Ghaffari, 2010), whose found that the erythroid lineage and HSCs are the most susceptible haematopoietic cells influenced by oxidative stress due to accretion of ROS that to erythroid destruction and diminish the erythropoiesis. This viewpoint informs the findings of a study that found an opposite relation between the genes for heat shock proteins and antioxidant enzymes and the number of blood cell progenitor cells of both myeloid and erythroid cells. This indicates when antioxidant levels go up and Hsps expression starts to rise, the number of these cells declines, which could be due to an excess of ROS formation,

which leads to cellular damage accumulation and activation of senescence mechanisms as a result of ROS development. Inspite of the negative effects of stress and rising ROS levels in HSCs, ROS are essential for normal haematopoiesis due to their strict regulation of differentiation and self-renewal capacity. This is agreement with the predictions of (Tothova *et al.*, 2007), who explored that the transcription factor FoxO is the most significant and influential regulator of free radicals in SCs through upstream regulatory oversight of its target antioxidant gene GPX3 in the hematopoietic system without adversely impacting the myeloid lineage. While downstream regulation of all FoxO isoforms leads to a reduction in SCs as well as a boost in ROS. Moreover, another supportive study was (Stahnke, 2016), whose found that GPX4 evidently upregulated in the SCs, and HSCs compared to other bone marrow cells and that HSCs promote long-tem protection against cellular stress in strict niche hypoxia leading to the overexpression of ROS-detoxifying enzymes supplying more protection for HSCs. Simultaneously (Stahnke, 2016), he observed that the upstream expression of GPX4 may have more pathways to preserve HSCS from oxidative stress in order to sustain their stemness and self-renewal capacity regardless of lineage or maturation.

Overall discussion, the haematopoiesis during the different stages of life is subject to many internal and external influences, but internal factors take the largest part of this influence. These factors are vital to the maintenance and behavior of bone marrow cells, and their impact in limiting their function is observed in the cellular signals that support and enforce the activity of these cells. The reactive oxygen species produced by different metabolic processes of stem cells, haematopoietic cell progenitors, and non-haematopoietic cells in the bone marrow are the most important of these

intrinsic factors that play a key role. These ROS arise due to different levels of hypoxia in the stem cells themselves, which would cause direct or indirect damage to the cells. At the same time, these ROS are very important in the activity of cells, but within specific levels which are critical in maintaining normal hematopoiesis and quiescent state with necessary rate of self-renewal. At physiological levels, these ROS serve as molecular signalling mediate different cellular response for instance, cell differentiation, proliferation, survival, migration and gene expression. Moreover, it is extremely important during the stage of haematopoiesis, there are different cellular responses within HSCs in terms of stimulating a specific protective system from unwanted effects, for example, stimulating heat shock proteins of different types to protect new protein synthesis by changing their shape by increasing the number of folds or reducing the number of folds of un/miss-folded proteins. It folds in a way that ensures that the new protein performs the same function as the original protein. The upregulation of ROS fosters the defense system of cellular antioxidant enzymes, which also play a key role in removing these free radicals on the one hand and as secondary messengers important in the transmission of cellular signals on the other, as well as the role of free radicals as a secondary messenger. Combined, all of these factors operate within a highly interdependent and complex microenvironment in order to maintain the function of HSCs, SCs and progenitor cells in a highly interdependent manner to keep their stemness, self-renewal, differentiation, proliferation, maturation, and survival. Free radicals are one of the most important internal signals related to oxygen levels, which interfere with the functions of many internal biosystems and by regulating their activity, being the link between all internal factors, which acts to balance the function of heat shock proteins on the one hand and to

balance antioxidant enzymes on the other hand through regulating expression of the coded genes that regulate these factors. Although of the detrimental effect of intracellular stress and increased ROS level in HSCs, the existence of ROS is very crucial for physiological haematopoiesis with their strict regulation on differentiation and self-renewal capacity via different cellular pathways.

Chapter six

The conclusion and recommendations

The conclusions

Overall, the study concludes several points which are the following;

1. Bone marrow cells differentiation and proliferation are related to age progress independently.
2. Haematopoietic cells differentiation and proliferation are regulated by bone marrow microenvironment stress conditions and by expression different Hsps and antioxidant genes.
3. Hsps90α and 90β and antioxidant enzymes CAT, SOD3 and GPX1 are the main genes that regulate the myeloid cell line production and differentiation.
4. Hsps90β and 27 and antioxidant SOD3 are mainly regulates the erythroid cell line production and differentiation and maturation.
5. A direct relationship between Hsp90β and SOD3 and inverse relationship between GPX1 and Hsps90α was noticed. While there is no correlation between all antioxidant genes and Hsp27 gene expression during haematopoiesis.
6. The presence of ROS in niche and HSCs, SCs, progenitor cells is very crucial for physiological haematopoiesis via their strict regulation on differentiation and self-renewal capacity via different cellular pathways.

The recommendations

Based on our findings the recommends several points for future works;

1. Studying the haematopoiesis relationship with mitochondrial haematopoietic cell activity.
2. Figuring-out the role HIF α and β in bone marrow niche regulation.
3. Studying the gene expression profiling of nd1 gene and its relation to Hsps 90β in bone marrow cells.
4. Studying haematopoiesis relationship with gene expression profile during late stage of embryogenesis.

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الخلاصة

لا تكمن أهمية نخاع العظام في تزويد الجسم بخلايا الدم المختلفة فحسب، بل تكمن في قدرته على تزويد الجسم بالخلايا الجذعية المتجددة التي تشارك في تجديد الأنسجة أو زرع الخلايا الجذعية كعلاج للعديد من الأمراض. يتم تنظيم خلايا نخاع العظام من خلال العديد من العوامل الجوهرية، وأهمها بروتينات الصدمة الحرارية والإنزيمات المضادة للأكسدة التي تعمل معا داخل بيئة دقيقة للحفاظ على عمل هذه الخلايا أثناء نمط الحياة. كان الهدف من الدراسة هو النظر في العلاقة بين تنميط التعبير الجيني في الخلايا المكونة للدم نخاع العظام للجرذان السوية من خلال دراسة التعبير الجيني لبعض بروتينات الصدمة الحرارية والإنزيمات المضادة للأكسدة، وتقييم الخلايا نخاع العظام في غضون الأشهر الثلاثة الأولى من العمر. تم استخدام تسعين جروا حديثي الولادة خلال فترة الدراسة، مقسمة إلى ثلاث مجموعات وفقا لتصميم الدراسة ثلاثين جروا لكل منهما. مجموعة الأولى تم الاحتفاظ الجراء حتى عمر شهر، المجموعة الثانية تم الاحتفاظ الجراء حتى عمر شهرين وتركت المجموعة الثالثة حتى ثلاثة أشهر من العمر. عندما وصلت الجرذان إلى العمر المحدد، تم أخذ عينات نخاع العظم بعد الموت الرحيم للحيوانات بالطريقة الفيزيائية للقتل الرحيم عن طريق خلع الفقرات العنقية للحيوانات غير المخدرة. تم جمع عينات نخاع العظم من عظم الفخذ والساق. تم عمل مسحات نخاع العظام وصيغها بصبغة May Grunwald-Giemsa لتقييم خلايا نخاع العظم. ثم تم جمع كل 5 عينات في أنبوب اختبار إيبندورف، 6 أنابيب لكل مجموعة، وحفظت في التجميد في −80 درجة مئوية لدراسة التعبير الجيني للجينات المختارة.

أظهرت نتائج مسحات نخاع العظم مراحل مختلفة من تكون الدم بما في ذلك إنتاج خطوط خلايا الكريات الحمر والبيض والخلايا العملاقة المكونة للصفيحات الدموية. علاوة على ذلك، كشف التقييم الشكلي للخلايا المكونة للدم عن جميع الأنواع المختلفة من الأسلاف والخلايا غير الناضجة لكل من خلايا الدم الحمراء والخلايا النخاعية وتنوعت أعدادهم مع تقدم العمر.

وعلاوة على ذلك، أظهر التعبير الجيني لبروتينات الصدمة الحرارية والإنزيمات المضادة للأكسدة التنظيم المستمر في جميع أنحاء الدراسة باستثناء بروتين الصدمة الحرارية 90، حيث كشفت تنظيم واطئ في عمر 3 أشهر مقارنة مع الشهر الأول والثاني، وكشفت التعبير الجيني لمضاد للأكسدة كلوتاثيون بيروكسديز 1 عن التنظيم العالي في الشهرين الأول والثاني من العمر مقارنة مع الشهر الأول من العمر. بالإضافة إلى أن تغيير عدد طيات بروتين الصدمة الحرارية 90 بيتا أظهر زيادة في تغيير عدد طيات البروتين طوال فترة الدراسة، في حين أظهر بروتين الصدمة

الحرارية90 ألفا زيادة مضاعفة في تغيير عدد الطيات في الشهر الثالث من العمر. كما أظهرت إنزيمات مضادات الأكسدة زيادة في تغير عدد الطيات طوال فترة الدراسة.

كشفت العلاقة بين الجينات المدروسة عن علاقة قوية مباشرة بين أنزيم السوبر أوكسيد ديسميوتيز 3 وبروتين الصدمة الحرارية 90 بيتا وارتباط عكسي قوي بين أنزيم الكلوتاثيون بيروكسيديز 1 والتعبير الجيني لبروتين الصدمة الحرارية 90ألفا فقط. في حين أن الجينات الأخرى أظهرت ارتباطا خفيفا غير معنوي. وفي الوقت نفسه، في النظر في العلاقة بين تنميط التعبير الجيني والخلايا المكونة للدم نخاع العظام السليمة كانت مثيرة للاهتمام من خلال ارتباطها فيما بينها بطريقة مستقلة وفقا لنوع من الخلايا المكونة للدم وبعض الجينات.

وخلصت الدراسة إلى أن تمايز خلايا نخاع العظام وانتشارها يرتبطان بالتقدم في العمر، ويتم تنظيم تمايز الخلايا المكونة للدم وانتشارها من خلال ظروف البيئة الدقيقة لنخاع العظام عن طريق جينات بروتينات الصدمة الحرارية وجينات الانزيمات المضادة للأكسدة. أيضا، بروتيني الصدمة الحرارية 90 ألف وبيتا والإنزيمات المضادة للأكسدة الكاتاليز، السوبر أوكسيد ديسميوتيز 3و الكلوتاثيون بيروكسيديز 1 هي الجينات الرئيسية التي تنظم إنتاج وتمايز خط الخلايا النخاعية البيضاء. بروتين الصدمة الحرارية 90 بيتا و27 وأنزيم مضاد الأكسدة السوبر أوكسيد ديسميوتيز هي أساسا لتنظم نضج وتمايز إنتاج خط خلايا الدم الحمراء. وقد لوحظت علاقة مباشرة بين بروتين الصمدة الحرارية 90 بيتا وأنزيم السوبر أوكسيد ديسميوتيز 3 وعلاقة العكسية بين أنزيم الكلوتاثيون بيروكسيديز 1 و بروتين الصدمة الحرارية 90 ألفا. وعدم وجود علاقة بين جميع جينات الأنزيمات المضادة للأكسدة والتعبير الجيني لبروتين الصدمة الحرارية 27 خلال عملية تكون الدم.