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Heat shock protein and antioxidant enzyme gene expression and fold changes in bone marrow samples from healthy rats

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Abstract

The hematopoiesis in the bone marrow in the early stages of life is very complex and involves many cellular factors under very strict micro-environments. The two important cellular factors are heat shock proteins and antioxidant enzymes. The aim of the study was to study the gene expression profile of healthy rats' bone marrow cells and niches particularly heat shock proteins and antioxidant genes. A total of thirty healthy Wister Albino rats at one, two, and three months old were involved throughout the study. Bone marrow samples were collected at a specified date according to the study design and utilized for qPCR test for expression of Hsps27, 90α, 90β, and antioxidant enzymes glutathione peroxidase1 (GPX1), catalase CAT and superoxide dismutase (SOD3) genes. The results revealed that there was a clear expression of both Hsps90α andβ in bone marrow cells throughout the study as well as the GPX1 gene. Furthermore, there was an increase in fold change of Hsps90α and β as well as GPX1, CAT, and SOD3 proteins as age progressed. In conclusion, hematopoietic cell differentiation and proliferation are regulated by bone marrow microenvironment stress conditions and by the expression of different Hsps and antioxidant genes. The protein folding process is a defense mechanism to protect the HSCs, and progenitors from un/misfolded proteins and to keep proteostasis.

Keywords: bone marrow cytology, bone marrow niche, gene expression, Hsps, antioxidants

التعبير الجيني لبروتينات الصدمة الحرارية والأنزيمات المضادة لأكسدة وتغير الطيات في عينات نخاع العظم للجرذان السليمة

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الخلاصة

تعد عملية تكوين الدم في نخاع العظام في المراحل المبكرة من الحياة معقدة للغاية وتتضمن العديد من العوامل الخلوية في ظل بيئات دقيقة شديدة الصرامة. تعتبر بروتينات الصدمة الحرارية والإنزيمات المضادة للأكسدة أهم عاملين خلويين داخل الخلية التي تدخل في عمل وتنظيم تكوين خلايا الدم. كان الهدف من الدراسة هو دراسة ملف التعبير الجيني لخلايا نخاع العظام للفئران السليمة وخاصة الجينات بروتينات الصدمة الحرارية والجينات المضادة للأكسدة. استخدم ثلاثون جرذا أبيض اللون سليما في عمر شهر، شهرين، وثلاثة أشهر في

الدراسة. تم جمع عينات نخاع العظام في تاريخ محدد وفقًا لتصميم الدراسة واستخدامها لاختبار تفاعل البلمرة المتسلسل الكمي qPCR للتعبير عن جينات Hsps27 وα90 وβ90 ومضادات الأكسدة GPX1 وCAT وSOD3. أظهرت النتائج أن هناك تعبيرًا واضحًا عن كل من Hsps90α وβ في خلايا نخاع العظم طوال فترة الدراسة بالإضافة إلى جين GPX1. علاوة على ذلك، كانت هناك زيادة في عدد طيات بروتينات Hsps90α وكذلك GPX1 وCAT وSOD3 مع تقدم العمر. استنتج من الدراسة أن تنظيم تمايز الخلايا المكونة للدم وتكاثرها عن طريق ظروف إجهاد المحيط الدقيق لنخاع العظم والتعبير عن جينات بروتينات الصدمة الحرارية المختلفة ومضادات الأكسدة. عملية طي البروتين هي آلية دفاعية لحماية الخلايا الجذعية والأسلاف من البروتينات غير المطوية وللحفاظ على التوزان البروتيني الخلوي.

الكلمات المفتاحية: نخاع العظم، التعبير الجيني، بروتينات الصدمة الحرارية، مضادات الأكسدة

Introduction

The process of formation, development, and differentiation of bone marrow cells in the early stages of life is very complex and involves many cellular factors under very strict micro-environments. The most prevalent factors are heat shock proteins (Hsps), which play a crucial key role in mesenchymal stem cell (MSCs) differentiation [1]. Hsps function as molecular chaperones that stabilize intracellular proteins, repair damaged proteins, protect existing proteins from aggregation and mediating the folding of newly translated proteins [2]. Hsps expression is effectively induced by cellular stress. This stress response and pluripotency maintenance has been shown in a variety of stem cell types, including adult stem cells, induced pluripotent stem cells, and embryonic stem cells [3]. Activation of the heat shock transcription factors (HSFs) triggers this stress response, resulting in higher Hsps transcriptional regulation. [4]. It's worthwhile to know that specific Hsps are strongly expressed in SCs and interact with distinct transcription factors to promote appropriate cell growth and function. [5]. The strict regulation of SC self-renewal, differentiation, survival, and aging is undoubtedly influenced by a variety of intrinsic and extrinsic signals; nonetheless, mounting data suggests that Hsps are crucial to the regulation of many types of SCs. [6 and 7]. A plethora of evidence suggests that the right kind of stress accelerates the division of MSCs or progenitor cells, resulting in the release of HSPs such as small and large molecular mass proteins [8]. The behavior of stem cells, such as self-renewal, diversification, responses to environmental stress, and aging, is said to be governed by fluctuations in Hsp expression, which really is noteworthy. [9]. The triggering of spontaneous ROS from mitochondrial oxygen phosphorylation, a typical aspect of cell metabolic mechanisms, triggers mitochondrial dysfunction in bone marrow gradually. [10].

Diverse bioactive components are required for signal conduction and energy consumption during cell growth; as a combination of these two mechanisms, ROS levels are significantly elevated. Hematopoietic stem cells (HSCs) exhibit unique cell cycle phases involving fluctuating ROS levels, therefore modulating their physiological activities. [11]. Likewise, elevated ROS levels in MSCs and HSCs drive HSC recruitment and translocation. Conversely, once ROS levels go up excessively high, HSCs potentially initiate a defensive mechanism to stop performing self-renewal. [12]. Due to the excessive of ROS triggering mitotic arrests and mutation, a protective network has evolved to scavenge ROS and restore the redox homeostasis in cells. [13]. To summarize, superoxide dismutase (SOD) transforms incremental O2 compounds in cells into H_2O_2. Although catalase and/or glutathione peroxidase (GPX1) can quickly convert H2O2 to safe water, H2O2 is usually toxic to cells. However, if the cells lack catalase and GPX1 to detoxify H2O2, the leftover H_2O_2 is transformed into even more deleterious OH-ions. [14]. SOD does seem to be a two-edged sword. Undoubtedly, it is well known that some antioxidants and prooxidants rely on the involvement of other antioxidant enzymes in cells, such as catalase and GPX1. A globalized controller of antioxidant system defense identified as Nrf2 was explored, and to date, a diversity of ROS-detoxifying enzymes, such as SOD, catalase, GPX1, and thioredoxin, have been discovered to somehow be governed by this regulator as downstream target genes. [15]. Increased ROS

levels have been proposed as one of the factors causing HSC defects in elderly mice, as discovered by Porto *et al* [16], who encountered that subcellular ROS levels in HSCs seemed to be considerably greater in the elderly than in the young. In murine models, "aging" HSCs demonstrated a high level of Cellular damage and high rates of senescence and/or apoptosis, and these higher levels of ROS have always been associated with more diverse HSCs [16]. Furthermore, BM stromal cells reduce BM cellularity significantly because they produce more ROS than HSCs. Considerably, steadily increasing ROS levels adversely affect the ability of BM stromal cells to facilitate HSC resumption in aged mice. [17]. This process is intimately associated to the steady progress of age. [18]. The aim of the study was to evaluate the gene expression of certain Hsps and antioxidant enzymes in bone marrow samples of healthy rats.

Materials and methods

Experimental Animals

Thirty healthy albino rats aged one, two, and three months were used in the study. Throughout the trial, tap water and a commercial pellet diet were provided ad libitum. Bone marrow samples were collected according to Maiti [19].

Bone marrow collection

According to the specified study times, when the age is completed, one month, two months, and three months, the rats were euthanized by physical method of euthanasia via cervical dislocation of unanesthetized animals [20]. Briefly, the euthanized animal was back-side extended, shaved, aseptically prepared for skin incision just on sideways back of the thigh, and both the tibia and femur were exteriorized after the muscular and tendinous relationships were stripped away. The metaphyseal region of both bones was trimmed with a scissor and a needle was inserted into the medullary cavity. The bone marrow was rinsed out using 5 ml of PBS in a petri dish, transferred to a centrifuge tube, then mixed by vortex, filtered by mesh, and washed-out bone marrow was then harvested by centrifugation in concentrating the cells for 10000 rpm/5 minutes. supernatant was decanted, and the sedimented cells were pipetted thoroughly before being used for qPCR analysis. Gene expression studies were conducted on bone marrow samples kept at -80° C.

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). Throughout brief, 5ml neutralized PBS was mixed with 5ml bone marrow samples from each group and separated by centrifugation at 13000 rpm for minutes to obtain the BM cells' pellet. The methodology was conducted in accordance with the manufacturer's instructions for RNA extraction. The reproduction of RNA to cDNA was then done by converting up to 5µg of total RNA or up to 500ng of RNA into first-strand cDNA using the GoScriptTM Reverse Transcriptase kit protocol. In summary, the procedure was divided into two stages. The first stage was to prepare the reverse transcription reaction mix by merging the ingredients of the GoScriptTM Reverse Transcription System in an aseptic microcentrifuge tube on ice. The next stage was to begin preparing and run the First-Strand cDNA Polymerization by first adding 15µl aliquots of the reverse transcriptase PCR mixture to each PCR reaction on ice, then adding 5µl of RNA and primer mix to each reaction for a total reaction mixture of 20µl per tube and incubating it for 30 minutes using a Biometra thermal cycler (Tprofessional® Basic 96, An Analytic Jena Company, Germany). The following protocol was used: Priming 5 minutes at 25 ºC. Reverse transcription at 42°C for up to 53 minutes, and RT inactivation 1 minute. At 95 ºC. finally, cDNA samples were kept in -20 ºC until further processing.

Running qRT-PCR

The gene expression of heat shock proteins and antioxidant enzymes mRNAs was investigated employing qRT-PCR on a Bioevapeak Real-Time PCR SystemTM Real-Time PCR System (China) and the SYBR green technique on a GoScriptTM Reverse Transcription System. The relative CT model was

used to calculate relative gene expression via relative qPCR (a number of cycles required for the fluorescent signal to cross the threshold). All materials were exposed to an RT-qPCR reaction in a total reaction mixture volume of 25µl, as follows: 12.5µl FastStart Universal SYBR Green Master (Rox), 1µl forward primer (20 pmol/l), 1-liter reverse primer (20 pmol/l), 2µl cDNA (templates), and 8.5µl sterile water. The RT-qPCR cycling conditions were as follows: an initial denaturation for three minutes at 95 oC, followed by 35 cycles of annealing 95 oC for one minute, and 60 oC for one minute, and extension at 72 oC for one minute. To compare the relative quantitative PCR, the Bioevapeak Real-Time qPCR Software (PCR-Q96-5) V. 2022 was in use, and the result calculated out of each specimen were normalized to β actin expression (housekeeping gene). The following factors have been considered using qPCR: Hsps (27, 90α, 90β) and antioxidant enzymes (GPX1, CAT, SOD3) mRNAs **β actin** *F 5'-TTG CCC TAG ACT TCG AGC AA-3', R 5'-AGA CTT ACA GTG TGG CCT CC-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'*, **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'*. which supplied by integrated DNA technologies, Singapore.

Data analysis

IBM SPSS Statistics 22 was used to analyze data (SPSS In. Chicago, IL., USA). Gene expression results were expressed as mean standard error (S.E). One-way analysis of variance (ANOVA) is also used to examine the data. The variability among studied groups were performed using a significance level less than 0.05, which has been defined to be proportionally significant as compared to 1 month old rats.

Results

The study of the gene expression of various genes of heat shock proteins and antioxidant enzymes revealed a significant difference in the levels of the studied genes in the bone marrow samples from healthy rats, as shown in figure (1). The following are the results of this study analyzed based on the cycle threshold (ct) value and the number of fold change for these genes:

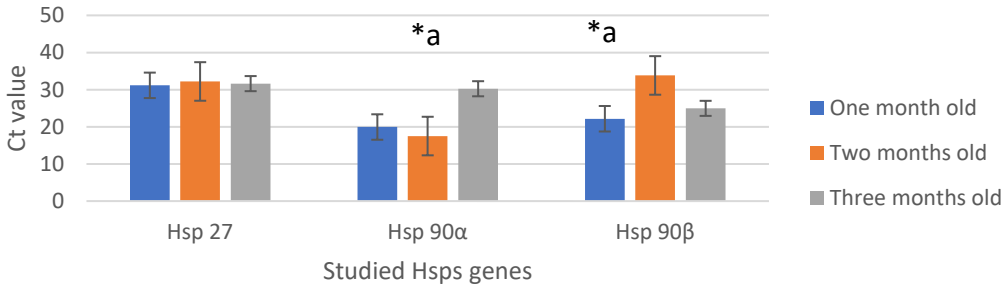

Figure (1) Effect of age on Hsps genes expression

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

Hsp 27 gene regulation was observed equally during the first three months, despite the fact that there was a low fold change in two months old studied rats, reaching 0.209, compared to the first and third months, which varied between 1.590-1.372-fold change with downstream regulation, as shown in figure (2).

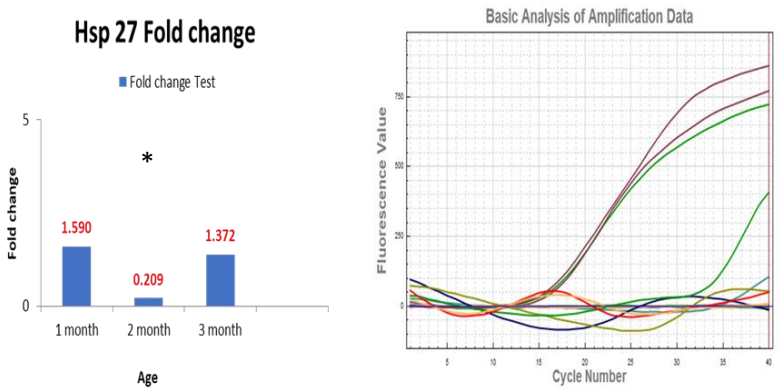

Figure (2) Effect of age on Hsp27 fold change and gene expression

Hsp 90 α expression increased up to 24-fold during the age of three months compared to one and two months, as shown in figure (3).

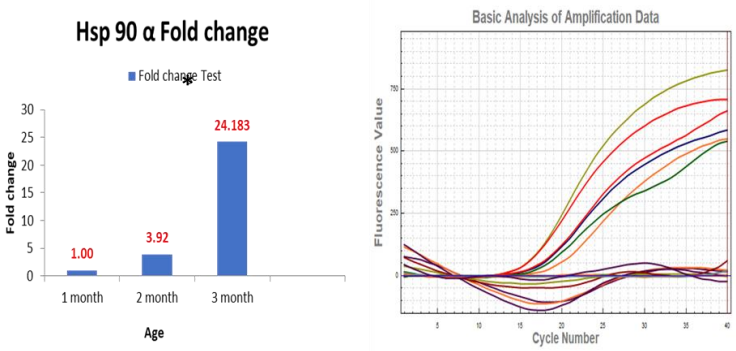

Figure (3) Effect of age on Hsp 90α fold change and gene expression

Despite its upstream regulation, Hsp 90β gene expression in healthy rat bone marrow cells did not vary in fold change during the first three months, with fold changes of 9.6, 14.6, and 12.5 respectively (figure 4).

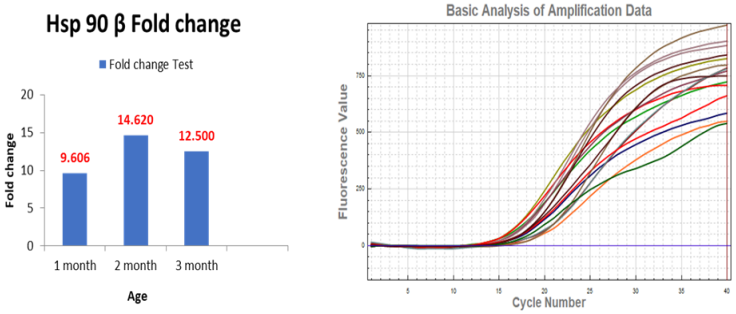

Figure (4) Effect of age on Hsp90β fold change and gene expression

GPX 1 mRNA expression in healthy rat bone marrow cells was significantly upstream regulated at two and three months compared to one month at $P \leq 0.05$. While the expression of CAT and SOD 3 enzymes remains constant with no variation during the first three months of life, figure (5).

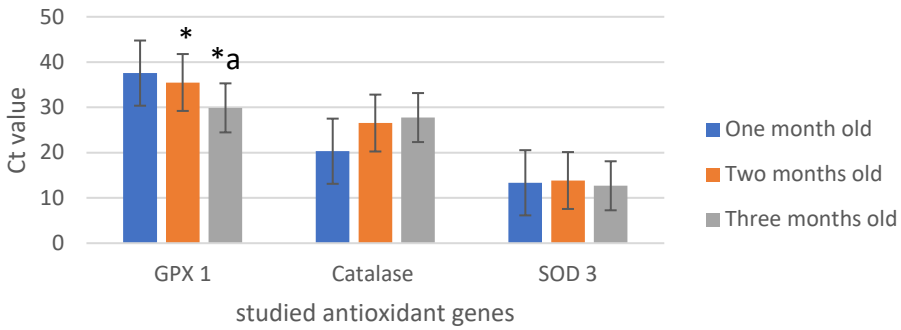

Figure (5) Effect of age on antioxidant genes expression

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

There was a tiny but discernible increase in the number of folds of the GPX1 enzyme in bone marrow samples of rats at the ages of 2 and 3 months compared to the first month of life, despite a decrease in these folds compared to the rest of the samples (figure 6).

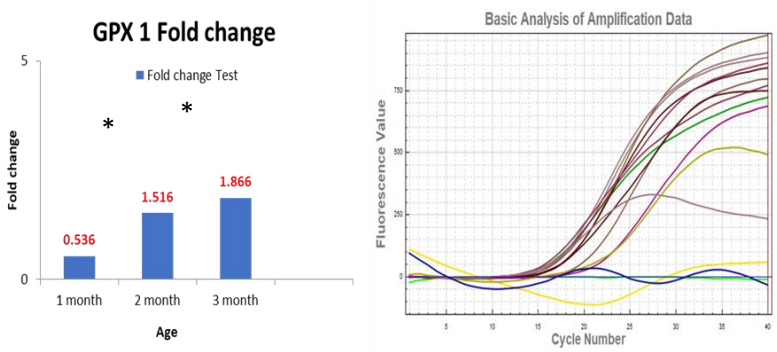

Figure (6) Effect of age on GPX1 fold change and gene expression

There was an increase in the number of CAT protein folds change in 2-month-old rat bone marrow samples (746.428), followed by a small but significant decrease in the number of folds change of the same enzyme in 3-month-old (523.483) samples compared to the number of CAT-folds change in 1-month-old rat hematopoietic stem cells (326.109), figure (7).

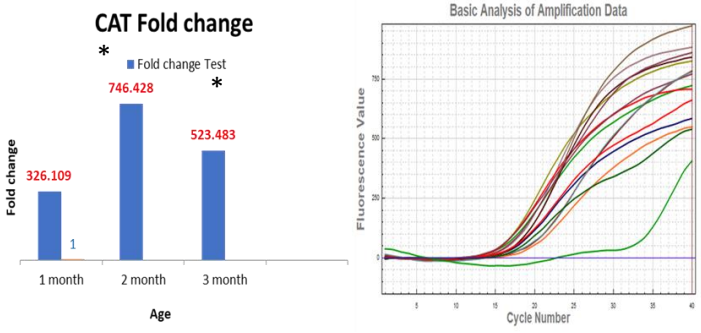

Figure (7) Effect of age on CAT fold change and gene expression

As shown in the figure 8, analysis of SOD 3 gene expression data revealed a double increase in the number of folds increases with age in the progenitor cells of the study rats.

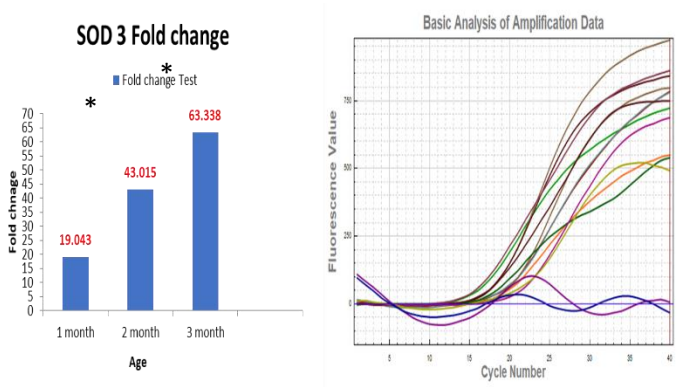

Figure (8) Effect of age on SOD3 fold change and gene expression

Discussion

The current work designed to study of the gene expression of a number of genes of Hsps as a cellular chaperon to protect any change in the nature of cellular proteins regulating the function and differentiation of HSCs in the bone marrow, as well as studying the gene expression of some genes regulating cellular antioxidant enzymes. These antioxidants effectively contribute to the homeostasis of the microenvironment of bone marrow cells, which plays a distinctive role in the formation and differentiation of HSCs [21]. In current study gene expression of different Hsps was very clear in bone marrow cells, this lead to understand the role of these genes in the maintenance of the equilibrium between activation and quiescence of different progenitors in the bone marrow via its role in niche. 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 [22] 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[23]. This supports the results of the current study in increasing the number of folds of heat shock proteins that play a vital and regulatory role in the bone marrow cell environment, and the capacity of this proteostasis drop with the aging associated with protein aggregation leading to pathological conditions.

In general, the bone marrow niche works with certain range of stress as consequence to hypoxia which is very necessary to haematopoiesis [24]. Thus, this stress generates the production and expulsion of free radicals, which attack proteins and change their nature and to preserve these proteins, especially the newly formed ones from damage, the cell’s chaperones work to protect these proteins, particularly newly formed ones, from damage, the cell's chaperones work to protect these proteins by unfolded protein response, which occurs in the endoplasmic reticulum [23]. Another explanation for increased protein folds is that protein chaperones help the correct folding proteins perform their normal function within the HSC or progenitor cells. Accordingly, the cellular factors such as transcription factors in link with certain HSPs are needed to control normal function and self- regeneration of stem cells [25]. The current finding is in agreement with (Gao et al., 2014) who, mentioned that there is a link between heat shock protein 90 and one of the internal factors (STAT3/Hop) by modulating and activating this factor and thus HSP90 is a

basic and very important protein for self-renewal stem cells. Therefore, expression of chaperones is advantageous in arresting stemness of various SCs by reducing the detrimental effect due to environmental and internal stress factors within bone marrow niche. Likewise, our findings are in agreement with Baharvand *et al*.[26], which express that upregulation levels of Hsps and co-factors in SCs can apply a buffering response contra external and internal stressor, can provide a buffering response against external and internal stressors by extending their stemness. Also, Baharvand *et al*. [27] supported the research findings by observing a down-steam gene expression in SCs differentiation, which 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. The occurrence of oxidative stress in the stem cell in certain level leads to several cellular responses, including cell cycle arrest and programmed cell death, 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. According to the current findings, there is an increase in protein fold change in certain studied genes during the study. This could be due to protein synthesis, in which peptides are twisted into folds with the help of glycosylation and molecular chaperones.

Indeed, only in certain cellular stressful situations, such as oxidative stress, hypoxia, and endoplasmic reticulum overcapacity, can the folding system be defective, resulting in the initiation of un-folded and mis-folded proteins, as supported by [28]. Besides which, the identification of such unexpected proteins by cellular cofactors (Hsps) because of overcapacity of protein folding and deterioration outcomes in a buildup of folded change proteins, that further involves the activation of three distinct ER stress response pathways due to the seriousness of the stress, provoking diverse cell reactions. This viewpoint is also supported by Sigurdsson and Miharada [29] which demonstrates the three ER stress response pathways related to the severity of cellular stress, which are non-stressed condition, modest/transient stress, and robust/chronic stress, which lead to induce fold change proteins under regulation of certain cellular signals predominantly governed survival signals by promoting protein folding but decreasing misfolded/unfolded proteins. As a response to newly synthesized un/miss folded protein under certain conditions which is functionally and structurally irrelevant proteins that is essential for the appropriate folding of newly synthesized polypeptide chains and unstable protein conformers [30]. As a result of the nature of the niche requirement to maintain HSC differentiation and proliferation to a certain level of oxidative stress, current findings revealed an increase in antioxidant enzyme fold change, which is a 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 heat shock response. It is clear that the activation of antioxidant enzymes by increasing the gene expression of genes regulating the synthesis and function of these enzymes is closely related to the cellular activity of bone marrow cells, which function differently under specific conditions of cellular stress caused by the formation of active oxygen species.

Conclusion

The current study concludes that the haematopoietic cells differentiation and proliferation are regulated by bone marrow microenvironment stress conditions and by expression of different Hsps and antioxidant genes. Protein folding is a defense mechanism that protects HSCs and progenitors from un/misfolded proteins and maintains proteostasis.

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