Med. J. Cairo Univ., Vol. 84, No. 1, September: 1211-1217, 2016
www.medicaljournalofcairouniversity.net

Effect of Erythropoietin on Bone Marrow Derived Mesenchymal Stem Cells Growth and Proliferation

MOHAMED A. WASSEF, M.D.; LAILA A. RASHED, M.D.; AMIRA A. HASSOUNA, M.D.; RADWA TAHA, M.D. and TAHA AZOUZ, M.Sc.

The Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University

Abstract

Background: Bone marrow mesenchymal stem cells (BM-MSCs) can differentiate into osteoblasts, adipocytes, and other mesenchymal cell lineages. These cells can also be expanded in culture for long periods without any apparent loss of differentiation capacity. Erythropoietin (EPO), is a molecule produced by kidney, and stimulates the proliferation and differentiation of erythroid progenitor cells. EPO can stimulate the proliferation and protraction of endothelial progenitor cells, and plays an important role in the proliferation and differentiation of marrow-derived mesenchymal stem cells.

Objectives: We aimed to investigate the possible protective effect of EPO, especially anti-apoptotic effect on MSCs growth.

Methods: Isolated bone marrow-derived mesenchymal stem cells from rats were treated with 10 IU/mL EPO for 5 days the cells were examined for the following:

- Gene expression of caspase-3, BAX/BCL–2 ratio and STAT–5.
- Cell proliferation assay.

Results: Treatment of MSCs with EPO for only 5 days promotes MSCs growth and proliferation. EPO significantly increase gene expression of STAT-5 and BCL2 while significantly decrease gene expression of BAX andcaspase-3 with decreasing BAX/BCL2. EPO also increase cell proliferation assay.

Conclusion: Through anti-apoptotic effect, EPO promote MSCs growth and proliferation.

Key Words: *Erythropoietin (EPO) – Stem Cells – Mesenchymal Stem Cells (MSCs) – Apoptosis.*

Introduction

STEM cells hold the remarkable capacity of self-renewal and differentiation into more specialized cell lineages, and thus constitute a promising resource in regenerative medicine for the generation of appropriate cell types in cell replacement therapy [1]. Stem cell therapies provide unique opportunities for treating many diseases including that of the heart and brain, which have limited regenerative capacity [2].

Stem cells are a unique type of cells that form the basis of the development, growth and survival of a living organism. Though the term is often used to describe controversial embryonic stem cells, there are many different types of stem cells, classified by their original location and/or method of formation. Stem cells are undifferentiated cells that go on developing into any of more than 200 type of cells that adult human body hold [3].

Mesenchymal stem cells (MSCs) (also known as skeletal stem cells or bone marrow stromal stem cells) are plastic adherent, non-hematopoietic cells that reside in a perivascular niche in the bone marrow stroma, that possess self renewal and multi-lineage (multipotent) differentiation capacity [4]. In the 2006 International Society for Cellular Therapy position paper that conveyed a set of simple criteria for defining a culture of cells as a culture of MSCs. The criteria were; adherence to plastic; expression of CD44, CD105, CD90, CD73 receptors; lack of hematopoietic (such as CD14, CD31, CD33, CD34, and CD45) and endothelial markers; in vitro differentiation to cartilage, bone, and fat; chemical induction in nonclonal cultures [5].

Based on these criteria, isolation of MSC-like cells has been reported from different tissues other than bone marrow including adipose tissue, umbilical cord, dental pulp, skeletal muscle, periodontal ligament and even brain [6]. MSCs also has been isolated from peripheral blood, cord Wharton's jelly, amniotic fluid, compact bone, periosteum,

Correspondence to: Dr. Taha Azouz,
E-mail: t.azouz@hotmail.com

synovial membrane and synovial fluid, articular cartilage and foetal tissues [7]. Only bone marrow-derived MSCs have documented evidence of stemness including the ability to form bone and bone marrow organ upon serial transplantation in vivo [8].

According to multi-lineage differentiation potential, it was believed that MSCs mediated tissue and organ repair. MSCs strategically form niches in perivascular spaces in almost every region of the body. It is thought that such localization allows them to detect local and distant tissue damage, as in wound infliction, and respond by migration to these sites and promoting tissue repair and healing [9]. In the last 20 years, MSCs were isolated from a wide range of tissues and organs. Furthermore, it was demonstrated that under specific stimuli MSCs possessed an incredible capacity of trans-differentiation, developing in mesodermal (myocyte, osteocyte, endothelium, adipocyte, and cardiomyocyte), ectodermal (neuronal), and endodermal (hepatic, pancreatic, respiratory epithelium) lineages [10].

Although the fascinating therapeutic effects of MSCs in cerebral and spinal cord injury, hematological disorders, cardiovascular diseases, diabetes, immune diseases, graft versus host diseases and cancer are well documented, the in depth mechanisms of how MSCs act remain a matter for debate and exploration. The generally putative concepts cover transdifferentiation, cell fusion, paracrine effects, microvesicles carrying messenger RNA (mRNA) or micro RNA (miRNA) and mitochondrial transfer [11].

Human Erythropoietin (EPO) is an acidic glycoprotein with a molecular mass of 30.4kDa. Its 165 amino acid residues chain forms four antiparallel α-helices, two β-sheets and two intra-chain disulfide bridges [12]. The continuous formation of new red blood cells (RBCs) is regulated by the EPO. The nucleotide sequence of the human EPO cDNA was reported in 1985. Since then, human EPO has become a major therapeutic agent to treat anemia of chronic renal failure and other diseases. More recently, EPO has also been observed to protect endothelial, neural, cardiac, and other cell types against cytotoxic damage [13].

During fetal development, EPO is produced mainly by hepatocytes and interstitial cells of the liver. Following birth, the kidney (peritubular fibroblasts in the renal cortex) accounts for ~80% of EPO production [14]. EPO mRNA is also detectable in liver, spleen, bone marrow, lung and brain, and EPO may be translated in small amounts in these organs [12]. Aside from the kidney and liver, EPO and EPO-receptor (EPO-R) are expressed in the brain and in the cardiovascular, digestive, endocrine, female and male reproductive, and respiratory systems [15]. It is found that Neurons express EPO-R and astrocytes produce EPO [16].

Erythropoietin is essential for the survival, proliferation, and differentiation of erythrocyte progenitors in bone marrow. Erythrocyte production is continuously adjusted to regulate the loss of senescent red blood cells and to guarantee optimal tissue oxygenation [17]. EPO is a tissue-protective hormone with more pleiotropic potential than had previously been thought. Beside its essential role for survival, proliferation, and differentiation of erythrocyte progenitors in bone marrow, EPO carries non erythropoietic functions. More recently, EPO has also been observed to protect endothelial, neural, cardiac, and other cell types [13]. It can prevent the tissue destruction surrounding a site of injury by signaling via a non-hemopoietic receptor [18]. EPO also prevents apoptosis in reduced or absent oxygen tension, excitotoxicity, and free radical exposure [19].

Material and Methods

This work was performed at the Unit of Biochemistry and Molecular Biology at The Medical Biochemistry Department, Faculty of Medicine, Cairo University, and Cairo, Egypt between 2014 and 2015. Ethical committee approval was taken from our Faculty of Medicine, Cairo University.

Preparation of BM-derived MSCs:

Bone marrow was isolated and propagated according to the standard described method [20]. On day 14, the adherent colonies of cells were trypsinized, and counted. By optic and transmission electron microscopes, MSCs in culture were characterized by their adhesiveness and fusiform shape.

Flow cytometry identification of cells:

MSCs were washed and resuspended in phosphate-buffered saline. CD29, CD105 and CD34 all monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added directly to cells and kept for 1 hour in 4°C. The cells were then incubated with antimouse immunoglobulin G fluorescein conjugated secondary antibody (Millipore Corp, Temecula, CA) for 45 minutes on ice. Cell suspensions were washed twice and analyzed on a FACS caliber flow cytometer.

Treatment with erythropoietin:

By 14th day from start, MSCs growth was established. Cells were divided into 2 equal fractions, each fraction represent group. Each group more divided into 6 subfractions.

- First group was treated with 10 IU/mL EPO for successive 5 days.
- Second group served as a control group.

The following investigations were performed:

- Gene expression of caspase-3, BAX/BCL-2 ratio and STAT-5.
- Cell proliferation assay.

Total RNA was extracted from cell culture using QIAGEN® RNeasy® Micro system for Total RNA Isolation and purification (QIAGEN, Austin, Texas, USA). The total RNA (0. 5-2 µg) was used for cDNA conversion using high capacity cDNA reverse transcription kit (#K1 62 1, Fermentas, USA). Real-time qPCR amplification and analysis were performed using SYBR® Green PCR Master Mix Reagents Kit (Catalog Number 4309155) and Applied Biosystem Instrument with software version 3.1 (StepOneTM, USA). The qPCR assay with the primer sets were optimized at the annealing temperature (Table 1).

Table (1): The oligonucleotide primers sequence of studied genes.

	Primer sequence
Caspase -3	Forward primer : 5'- CTGGACTGCGGTATTG AGAC - 3'
	Reverse primer : 5'- CCGGGTGCGGTAGAGT AAGC - 3'
BAX	Forward primer : 5'- GTTGCCCTCTTCTACT TTG - 3'
	Reverse primer : 5'- AGCCACCCTGGTCTT G - 3'
BCL-2	Forward primer : 5'- CGGGAGAACAGGGTA TGA - 3'
	Reverse primer : 5'- CAGGCTGGAAGGAGA AGAT - 3'
STAT-5	Forward primer : 5'- ACCAACTTCCCCAACA TTCCT - 3'
	Reverse primer : 5'- ACTATGGCTGCCTGCC AGAA - 3'
GAPDH	Forward primer : 5' - TGCTGGTGCTGAGTA TGTCG - 3'
	Reverse primer : 5' - TTGAGAGCAATGCC AGCC - 3'

MTT cell proliferation assay:

Proliferation rate was assessed using Trevigen's TACS® MTT Cell Proliferation Assay (Gaithersburg MD 20877).

Statistical analysis:

Data were coded and entered using the statistical package SPSS version 22. Data was summarized using mean ± standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons bonferroni post hoc test. Correlation was done to test for linear relations between quantitative variables by Pearson correlation coefficient. p-values less than 0.05 were considered as statistically significant.

Results

The study showed the following results:

Table (2): Comparison between variables measured in the two studied groups.

Items	Non-EPO treated group Mean ± SD	EPO treated group Mean ± SD (RQ)	p-value
STAT-5 (RQ)	1.17±0.22	7.68±1.76	<0.001**
BAX (RQ)	1.08±0.07	0.17±0.06	<0.001**
BCL-2 (RQ)	1.06±0.07	9.00±2.13	<0.001**
Caspase-3 (RQ)	1.15±0.25	0.18±0.07	<0.001**
BAX/BCL-2	1.02±0.09	0.02±0.01	<0.001**
Proliferation (Cell n/mL)	84.83±3.71	95.33±3.56	0.001*

*: Significant (p<0.05). **: Highly significant (p<0.001). Levels are expressed as mean ± SD.

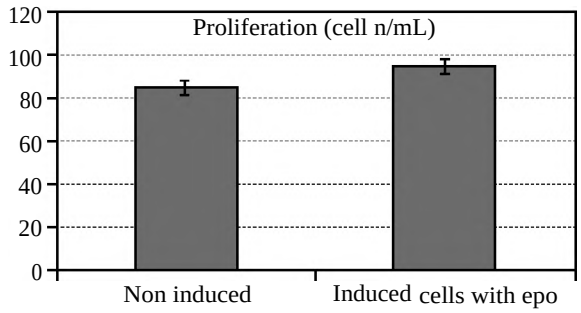


Fig. (1): Comparison of proliferation assay between the two studied groups.

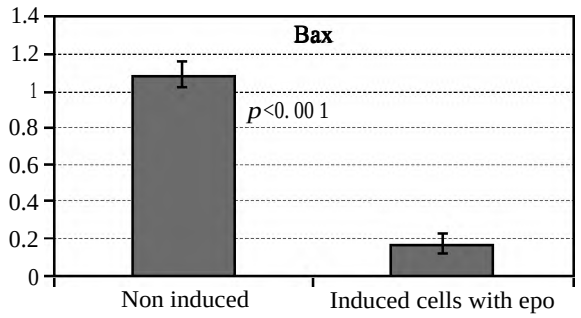


Fig. (2): Comparison between BAX gene expression of the two studied groups.

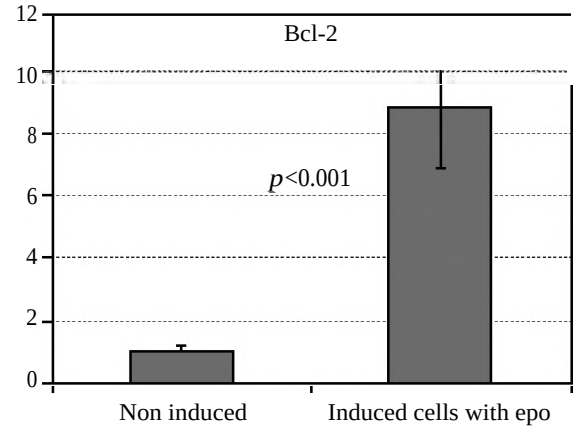


Fig. (3): Comparison between BCL-2 gene expression of the two studied groups.

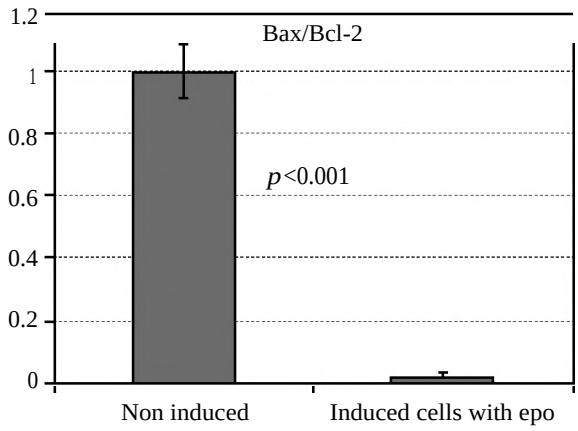


Fig. (4): BAX/BCL-2 ratio of the two studied groups.

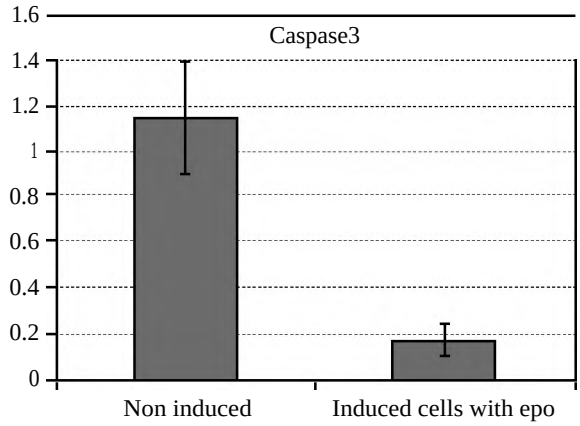


Fig. (5): Comparison between Caspase-3 gene expression of the two studied groups.

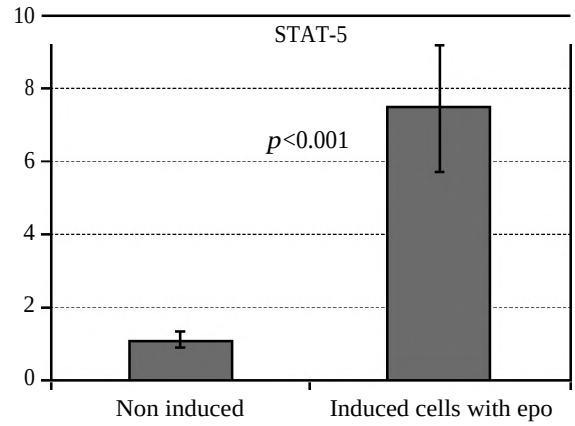


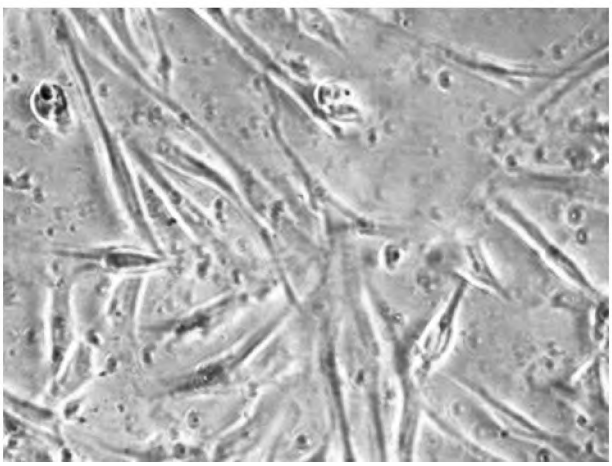
Fig. (6): Comparison between STAT-5 gene expression of the two studied groups.

In comparison to non-EPO treated group, EPO treated group shows:

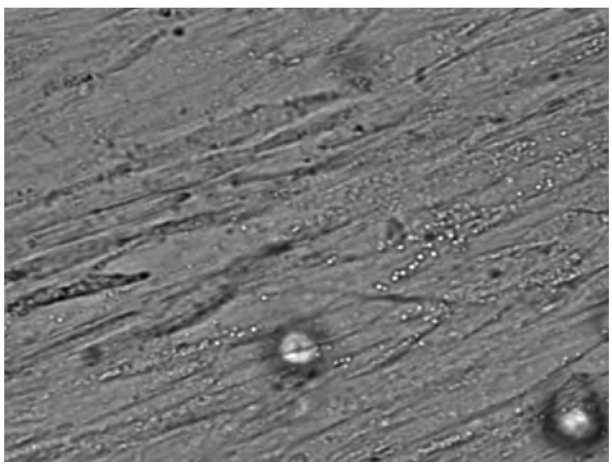
- Significant increase in gene expression of STAT-5 and BCL-2 & significant decrease in gene expression of BAX and Caspase-3.
- Significant increase in proliferation assay & significant decrease in BAX/BCL-2 ratio (Table 2 & Figs. 1-6).

MSCs Isolation and identification:

A- *MSCs in culture:*



(A)



(B)

Fig. (7): (A): Spindle shaped MSCs at one week culture, (B): MSCs at 2 weeks culture.

B- *Analysis of MSCs based on cell surface marker expression:*

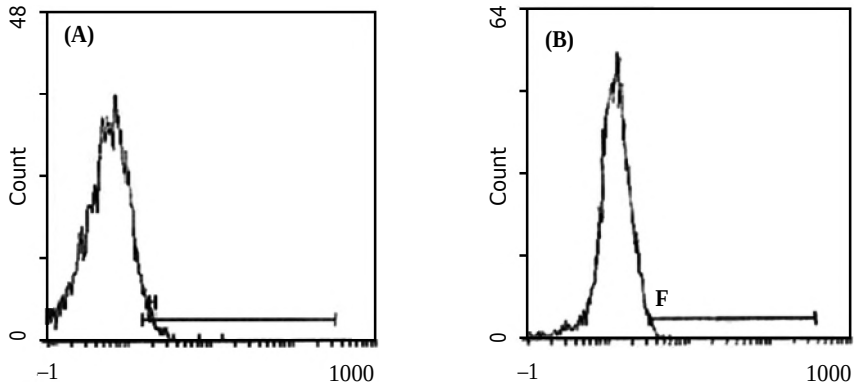


Fig. (8): Flow cytometric characterization analyses of bone marrow-derived MSCs.

Discussion

MSCs represent a hopeful gate in protective and treatment strategies through many mechanisms including engraftment property, paracrine secretion activity and transdifferentiation capacity [21]. Apoptosis is programmed cell death characterized by cytoplasmic condensation and nuclear fragmentation. Apoptosis signaling pathways fall under two categories: receptor-mediated apoptosis and mitochondrial-dependent apoptosis [22]. BCL-2 is a 26-kDa protein located in mitochondrial membranes; over expression of BCL-2 inhibits apoptosis [23].

In contrast, BAX, a cytosolic protein in normal living cells, can induce apoptosis and quickly translocates to mitochondria at an early stage of the apoptotic process. The anti-apoptotic effect of BCL-2 hinders the activity of the BAX pro-apoptotic protein. The ratio of BAX and BCL-2 in the outer membrane of the mitochondrion is a determining factor for the release of cytochrome C, and its binding to cytosolic Apaf-1 (apoptotic protease activating factor 1) and pro-caspase 9 to form complexes activates caspase9, which then activates caspase3 and ultimately initiates apoptosis [24].

EPO inhibits apoptosis by increasing anti-apoptotic factors such as BCL-2, BCL-XL, XIAP, (Hsp70s) that inhibit apoptosis through the mitochondrial membrane consolidation and preventing the entrance of cytochrome C into the cytoplasm and inhibit caspase triggering apoptosis [25]. EPO treatment showed anti-apoptotic effect not in vivo only, but also in vitro. The anti-apoptotic function of EPO has been clearly demonstrated. EPO-treated cell culture revealed down-regulation of apoptotic genes e.g. BAX and caspase-3 and up-regulation of anti-apoptotic genes BCL-2 and STAT5, thus increase BCL-2/BAX ratio which is considered a good indicator of apoptosis.

Signal transducer and activator of transcription STAT5 is an essential regulator of cell differentiation, proliferation and survival. Following stimulation with specific cytokines, growth factors and hormones, the latent transcription factor is phosphorylated by receptor-associated JAK tyrosine kinases [26]. STAT5 constitutive activation results in increased cell proliferation and reduced cell apoptosis, and is as such an important player in cancer initiation and progression [27]. EPO increased phosphorylation of STAT5 and furthermore, EPO modulated the nuclear translocation of phospho-STAT5, which increased expression of BCL-XL and decreased levels of caspase-3 [28].

Looking through the upper results, EPO increase remarkably the proliferation assay of EPO-treated cell culture compared to non EPO-treated group. EPO may increase proliferation assay by up-regulation of STAT5, increase anti-apoptotic ratio BCL-2/BAX and by anti-inflammatory effect. EPO expresses tissue protection via inhibition of NF-icB activation. Moreover, EPO impairs the formation of pro-inflammatory factors such as TNF-a, IL-6, IL 12/IL-23 subunits and NO via inducible NO synthase (iNOS) by macrophages [29]. Others suggested that erythropoietin observed anti-apoptotic effects could be partially explained by the noted effects on the antioxidant enzyme SOD and glutathione which were significantly increased after erythropoietin treatment [30].

Conclusion:

Administration of EPO promotes MSCs growth and proliferation most probably through many mechanisms strongly approved, apoptotic profile correction.

References

1- LI D.: The special issue on stem cell biology. Cell Research, 23: 1-2, 2013.

2- NGUYEN P.K., RIEGLER J. and WU J.C.: Stem Cell Imaging: From Bench to Bedside. Cell Stem Cell, 14 (4): 431-444, 2014.

3- PRAMOD P.M., SAGAR K.S., AMEET M.M., et al.: Stem cells: A new paradigm periodontal regeneration. Int. J. Med. Res. Health Sci., 2 (2): 254-260, 2013.

4- BIANCO P., CAO X., FRENETTE P.S., et al.: The meaning, the sense and the significance: Translating the science of mesenchymal stem cells into medicine. Nat. Med., 19 (1): 35-42, 2013.

5- BIANCO P.: Don't market stem-cell products ahead of proof. Nature, 499, 255, 2013.

6- ORBAY H., TOBITA M. and MIZUNO H.: Mesenchymal stem cells isolated from adipose and other tissues: Basic biological properties and clinical applications. Stem cells Int., 461718, 2012.

7- AUGELLO A., KURTH T.B. and DE BARI C.: Mesenchymal stem cells: A perspective from in vitro cultures to in vivo migration and niches. Eur. Cell Mater, 20: 121-33, 2010.

8- SACCHETTI B., FUNARI A., MICHIENZI S., et al.: Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell, 131 (2): 324-336, 2007.

9- GLENN J.D. and WHARTENBY K.A.: Mesenchymal stem cells: Emerging mechanisms of immunomodulation and therapy. World Journal of Stem Cells, 6 (5): 526-539, 2014.

10- FARINI A., SITZIA C., ERRATICO S., et al.: "Clinical Applications of Mesenchymal Stem Cells in Chronic Diseases". Stem Cells International, 306573, 2014.

11- LIANG X., DING Y., ZHANG Y., et al.: Paracrine Mechanisms of Mesenchymal Stem Cell-Based Therapy: Current Status and Perspectives. Cell Transplantation, 23: 1045-1059, 2014.

12- JELKMANN W.: Physiology and Pharmacology of Erythropoietin. Transfus Med. Hemother, 40: 302-309, 2013.

13- WOGNUM B.: Erythropoietin mini review. www.stemcell.com, catalog #29013 (VERSION 3.0.0) MAY 2011, 2011.

14- BUNN H.F.: Erythropoietin Cold Spring Harb. Perspect Med., 3 (3): a011619, 2013.

15- ZHANG Y., WANG L., DEY S., et al.: Erythropoietin Action in Stress Response, Tissue Maintenance and Metabolism; Int. J. Mol. Sci., 15: 10296-10333, 2014.

16- ALNAEELI M., WANG L., PIKNOVA B., et al.: Erythropoietin in Brain Development and Beyond. Anatomy Research International, 953264, 2012.

17- LOMBARDERO M., KOVACS K. and SCHEITHAUER B.W.: Erythropoietin: A Multifunctional Hormone. Pathobiology, 78 (1): 41-53, 2011.

18- BRINES M. and CERAMI A.: Emerging biological roles for erythropoietin in the nervous system. Nat. Rev. Neurosci, 6: 484-494, 2005.

19- MAIESE K., CHONG Z.Z., HOU J., et al.: Erythropoietin: New Directions for the Nervous System. Int. J. Mol. Sci., 13 (9): 11102-11129, 2012.

20- ALHADLAQ A. and MAO J.J.: Mesenchymal stem cells: Isolation and therapeutics. Stem Cells Dev., 13: 436-448, 2004.

21- PRASAJAK P. and LEEANANSAKSIRI W.: Mesenchymal Stem Cells: Current Clinical Applications and Therapeutic Potential in Liver Diseases. J. Bone Marrow Res., 2 (137): 1-9, 2014.

22- RAJ P.V., NITESH K., PRATEEK J., et al.: Effect of Lecithin on d-Galactosamine Induced Hepatotoxicity Through Mitochondrial Pathway Involving Bcl-2 and Bax. Indian Journal of Clinical Biochemistry, 26 (4): 378-384, 2011.

23- LIU C., SHI Z., FAN L., et al.: Resveratrol improves neuron protection and functional recovery in rat model of spinal cord injury. Brain Res., 1374: 100-9, 2011.

24- YIN F., GUO L., MENG C.Y., et al.: Transplantation of mesenchymal stem cells exerts anti-apoptotic effects in adult rats after spinal cord ischemia-reperfusion injury. Brain Res., 1561: 1-10, 2014.

25- EIPEL C., HUBSCHMANN U., ABSHAGEN K., et al.: Erythropoietin as Additive of Htk Preservation Solution in Cold Ischemia/Reperfusion Injury of Steatotic Livers. Journal of Surgical Research, 173: 171-179, 2012.

26- PINZ S., UNSER S., BUOB D., et al.: Deacetylase inhibitors repress STAT5-mediated transcription by interfering with bromodomain and extra-terminal (BET) protein function. Nucleic Acids Res., 43 (7): 3524-45, 2015.

27- VALENTINO L. and PIERRE J.: JAK/STAT signal transduction: Regulators and implication in hematological malignancies. Biochem. Pharmacol., 71: 713-721, 2006.

28- MA R., HU J., HUANG C., et al.: JAK2/STAT5/Bcl-xL signaling is essential for erythropoietin-mediated protection against apoptosis induced in PC 12 cells by the amyloid β-peptide Aβ25-35. British Journal of Pharmacology, 171 (13): 3234-3245, 2014.

29- NAIRZ M., SONNWEBER T., SCHROLL A., et al.: The pleiotropic effects of erythropoietin in infection and inflammation. Microbes and Infection/Institut Pasteur, 14 (3): 238-246, 2012.

30- SHARAWY N., RASHED L. and YOUAKIM M.F.: Evaluation of multi-neuroprotective effects of erythropoietin using cisplatin induced peripheral neurotoxicity model. Exp. Toxicol. Pathol., 67 (4): 315-22, 2015.

الملخص العربى

بالنظر إلى الخلايا الجذعية الوسيطة وما تمتلكه من قدره فائقة على التجدد والتمايز بما يمكنها من خلق فرص واعدة لعلاج العديد من الأمراض المزمنة والمستعصية بما فى ذلك الأمراض التنكسية العصبية وأيضا الأمراض ذات الطابع الحاد مثل الفشل الكبدي الحاد. ومن أهم آليات المفترضة لعمل الخلايا الجذعية الوسيطة؛ التميز لخلايا فعالة والإنصهار داخل خلايا اخرى وعملها كغدة ذات افراز مجاور ونقل الميتوكوندريا وايضا امكانية ارسال حويصلات تحمل الأحماض النووية. يمتلك الإرثروبويتين – وهو هرمون واقى للأنسجة – العديد من الوظائف أكثر مما كان يعتقد سابقا. من المعرف دور الإرثروبويتين فى تكاثر ونضج كريات الدم الحمراء. ومعروف ايضا انه أكثر الأدويه فاعلية فى علاج الأنيميا بمختلف اسبابها خصوصا الناتجة عن الفشل الكلوى المزمن. يعتقد أن الإرثروبويتين يقوم بوقاية الأنسجة من خلال مكافحة موت الخلايا المبرمج، ونشاطة كمضاد للأكسدة وايضا مكافحة الالتهابات.

تم عمل هذه الدراسة فى المعمل وذلك بعد استخلاص الخلايا الجذعية الوسيطة من نخاع عظام الفئرن ثم زراعتها وتوفير الظروف المناسبة لنمو هذه الخلايا. وبمرور أربعة عشر يوما التأكد من وجود الخلايا الجذعية الوسيطة بطرق مختلفة. اتبع ذلك تقسيم الخلايا الى مجموعتين. وقد تم علاج أحدى هاتين المجموعين من الخلايا الجذعية الوسيطة المشتقة من نخاع عظام الفئران ب ١٠ وحدة دولية/مل من الإرثروبويتين لمدة ٥ أيام.

ثم خضعت المجموعتين لفحص ما يلى:

- التعبير الجينى ل (STAT-5، BCL-2، BAX، Caspase-3) باستخدام تقنية تفاعل البلمرة المتسلسل الكمى اللحظى وأيضا نسبة BCL-2/BAX.
- فحص تكاثر الخلايا.

أثبتت نتائج الدراسة الدور المهم للإرثروبيوتين فى الحماية من موت الخلايا المبرمج وذلك عن طريق تقليل نشاط جينى BAX وCaspase-3 وزيادة نشاط جينى BCL-2 (وبذلك تناقص نسبة BAX/BCL-2) وSTAT-5 مما ادى الى زيادة تكاثر الخلايا مقارنة بالخلايا التى لم تعالج بإرثروبيوتين. تثبت هذه الدراسة أن اسخدام كلا من الإرثروبويتين والخلايا الجذعية الوسيطة ربما يزيد من فرص العلاج بالخلايا الجذعية الوسيطة بما يقدمة الإرثروبويتين من تقليل موت الخلايا المبرمج.