The protective effect of bone marrow derived mesenchymal stem cells and erythropoietin on experimental acute hepatic injury in rats

MOHAMED A. WASSEF[1], MD medical Biochemistry and Molecular Biology; LAILA A. RASHED[1], MD medical Biochemistry and Molecular Biology; AMIRA A. HASSOUNA[1], MD medical Biochemistry and Molecular Biology; SAMIA GABAL[2], MD Pathology; RADWA TAHA[1], MD medical Biochemistry and Molecular Biology; AND TAHA AZOUZ[1*] , MSc medical Biochemistry and molecular Biology

[1]Medical Biochemistry and Molecular biology department, Faculty of Medicine, Cairo University, Cairo, Egypt

[2]Pathology department, Faculty of medicine, Cairo University, Cairo, Egypt

***Correspondence to:** TAHA AZOUZ, t.azouz@hotmail.com

Abstract

Background: Tissue-protective effect of erythropoietin (EPO) was confirmed in brain, myocardium, kidney and liver injury. Mesenchymal Stem Cells (MSCs), with its remarkable capacity of self renewal and differentiation, provide opportunities for treating many diseases including acute liver failure. Experimentally, acute liver injury can be induced by well-known and often used hepatotoxin, galactosamine (GalN).

Objectives: We aimed to investigate the possible protective effect of both EPO and MSCs on GalN-induced liver injury and trying to clarify their mechanism of action. **Methods:** One hundred, male adult white albino rats were divided into five groups each consists of 20 rats. Group 1: served as negative control group, Group 2: rats were injected once with galactosamine (GalN) (650mg/kg IP), Group 3: rats were injected once with MSCs (one million cells/rat IP) immediately after GalN injection, Group 4: rats were injected once with EPO (12 IU/kg IP) immediately after GalN injection, Group 5: rats will be injected once with MSCs treated with EPO (IP) immediately after GalN injection. IL-6, IL-10 levels and NFκb, iNOS, TLR4&BAX gene expression were assessed in liver tissue. ALT, AST, albumin and ammonia levels were assessed in serum. Histopathology was done.

Results: EPO and MSCs treatment corrected the toxic effect of GalN with significant increase in the mean level of IL-10 & albumin and significant decrease in the mean level of IL-6, (NFκb, iNOS, TLR4& BAX gene expression) and (ammonia, ALT& AST). The combination between MSCs and EPO enquired more protection against GalN toxicity.

Conclusion: MSCs and EPO as separate or combined therapy protect liver against GalN toxicity may be through immunomodulation and anti-apoptotic activities.

Keywords: Erythropoietin (EPO), Stem Cells, Mesenchymal Stem Cells (MSCs), Acute Liver Failure.

Introduction

Acute liver failure (ALF) arises from sudden and severe hepatic injury due to many causes. It is associated with high mortality and resource cost. In many countries it is the most frequent indication for emergency liver transplantation [1]. Numerous epidemiological studies have described ALF as a heterogeneous syndrome with different patterns, prognoses, and outcomes [2].

Galactosamine (GalN) is a well-known and often used hepatotoxin [3]. The mechanism for GalN-induced liver injury seems to be partly related to the immune system. Its toxic effects are connected with insufficiency of UDP (uracil nucleotides)-sugar "UDP-glucose and UDP-galactose", release of pro-inflammatory mediators (such as TNF- α from Kupffer's cells), loss of intracellular calcium homeostasis and altering uridine-pool in hepatocytes [4].

These changes affect cell membranes, organelles and the synthesis of proteins and nucleic acids. GalN, at higher dose, inhibits the energy metabolism of hepatocytes, destroys the enzymes involved in the transport of substrates to the mitochondria and modifies the phospholipid composition of membranes [5].

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 [6].

Bone marrow mesenchymal stem cells (BM-MSCs) can differentiate into osteoblasts, adipocytes, and other mesenchymal cell lineages. The hepatocyte differentiation capacity of human BM-MSCs (hBM-MSCs) has been characterized in vitro and in vivo. Some groups have already started transplanting autologous bone marrow cells into patients with chronic liver fibrosis or cirrhosis. However, little is known about the use of hBM-MSCs to treat acute liver failure in animal models or in human patients with ALF, even though such studies would be clinically important [7].

Materials 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 Sep. 2014 and June 2015. The work was in collaboration with pathology departments, faculty of medicine, Cairo University. 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 [8]. 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.

Labeling of stem cells with PKH26 dye

Undifferentiated and differentiated MSCs cells were labeled with PKH26 according to the manufacturer's recommendations, (Sigma-Aldrich, Saint Louis, Missouri, USA). Cells were injected intravenously into rat tail vein. After one week, liver tissue was examined with a fluorescent microscope to detect the cells stained with PKH26 dye to ensure homing and to trace the injected cells in the liver tissue.

Preparation of experimental animal

This study included one hundred male rats inbred strain (Cux1: HEL1) of matched weight (120-150gm). Animals were inbred in the Experimental Animal Unit, Faculty of Medicine, Cairo University. Rats were maintained according to the standard guidelines of Institutional Animal Care and Use Committee and after Institutional Review Board approval. Animals were fed a semi-purified diet that contained (gm/kg): 200 casein, 555 sucrose, 100 cellulose, 100 fat blends, 35 vitamin mix, and 35 mineral mix. Animals were divided into 5 groups each consists of 20 rats:

Group 1: served as negative control group (healthy control). Group 2: Rats were injected once with galactosamine (GalN) (650mg/kg IP) dissolved in saline to induce acute hepatic toxicity (Bigoniya et al., 2009). Group 3: Rats were injected once with MSCs (one million cells/rat IP) immediately after GalN injection. Group 4: Rats were injected once with EPO (12 IU/kg IP) immediately after GalN injection. Group 5: Rats will be injected once with MSCs treated with EPO (IP) immediately after GalN injection.

At the planned time (7 days), venous blood was collected from the retro-orbital vein from rats of all groups and animals were sacrificed by cervical dislocations. Then serum was collected. Liver tissue was harvested and divided into 3 portions, 1st one for QRT-PCR, 2nd one for Elisa tests and 3rd for histopathological and Immunohistochemical study.

- The following investigations were performed:

1- Gene expression of NFκB, iNOS, TLR4& BAX gene by quantitative Real Time Polymerase Chain Reaction (qRT-PCR).

2- Estimation of tissue Level of IL-6 and IL-10 by Elisa (Bioscience, Austria).

3- Histopathological assessment of rats liver.

- Assessment of liver functions by measurement of albumin, ammonia, ALT and AST levels in serum by Quantitative- Enzymatic- Colorimetric determination kit which was provided by SPINREACT, SPAIN.

Total RNA was extracted from liver tissue homogenate using SV Total RNA Isolation system (Promega, Madison, WI, USA). The total RNA (0.5–2 μg) was used for cDNA

conversion using high capacity cDNA reverse transcription kit (#K1621, 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 (StepOne™, USA). The qPCR assay with the primer sets were optimized at the annealing temperature (table 1).

Histopathological examinations

Liver samples for Histopathological examinations were collected and were fixed overnight in 40 g/L paraformaldehyde in PBS at 4°C. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness of the right lobes of the livers by slidge microtome then stained by hematoxylin and eosin and examination was done through the light electric microscope. Unstained positively charged slides were prepared from each paraffin block for immunostaining using monoclonal rabbit anti-human antibody (anti-COX-2, Lab vision, USA. and ultra-vision detection system) (HRP/DAB, Lab vision, USA).

Table 1: The oligonucleotide primers sequence of studied genes

	Primer sequence
NF*κ*B	**Forward primer:** 5′- GCTTACGGTGGGATTGCATT - 3′ **Reverse primer:** 5′- TTATGGTGCCATGGGTGATG - 3′
iNOS	**Forward primer:** 5′- GGGCCACCT TTATGTTTGTG - 3′ **Reverse primer:** 5′- CCTCAACCTGCTCCTCACTC -3′
TLR4	**Forward primer:** 5′ -GCCGGAAAGTTATTGTGGTGGT-3′ **Reverse primer:** 5′-ATGGGTTTTAGGCGCAGAGTTT- 3′
BAX	**Forward primer:** 5′- GTTGCCCTCTTCTACTTTG - 3′ **Reverse primer:** 5′- AGCCACCCTGGTCTTG - 3′
GAPDH	**Forward primer:** 5′ - TGCTGGTGCTGAGTATGTCG - 3′ **Reverse primer:** 5′ - TTGAGAGCAATGCCAGCC - 3′

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

Table 2*: *Relations between all measured variables in all groups.

	Group I (control)	Group II (GalN)	Group III (GalN + MSCs)	Group IV (GalN+ EPO)	GroupV (GalN + EPO + MSCs)
NFκB*(RQ)*	*1.16± .29*	*10.38± 1.45 **	*4.85± .73 *#*	*4.99± 1.21 *#*	*3.75± .89 *#*
iNOS*(RQ)*	*1.03± .04*	*13.32± 1.68 **	*5.27± 1.17 *#*	*5.75± 1.75 *#*	*3.63± .79 #*
TLR4*(RQ)*	*1.08± .15*	*10.32± 2.08 **	*5.00± .64 *#*	*4.57± .92 *#*	*2.64± 1.25 #*
BAX*(RQ)*	*1.07± .09*	*11.08± 1.75 **	*5.04± 1.40 *#*	*4.82± 1.10 *#*	*2.76± .90 #*
IL-6(pg/mL)	*31.39± 3.23*	*148.62± 19.17 **	*67.15± 15.67 *#*	*83.35± 18.68 *#*	*56.94± 14.66 *#@*
IL-10(pg/mL)	*134.33± 15.08*	*60.64± 17.13 **	*96.94± 8.90 *#*	*82.42± 11.60 *#*	*109.76± 12.19 *#@*
Albumin(g/dL)	*5.29± .45*	*2.56± .46 **	*3.28± .50 **	*3.21± .33 **	*4.06± .56 *#*
Ammonia(µg/dL)	*11.86± 1.97*	*74.42± 5.66 **	*37.90± 10.06 *#*	*41.76± 8.17 *#*	*21.72± 5.10 #$@*
ALT(U/L)	*27.14± 5.20*	*91.56± 6.59 **	*47.50± 7.27 *#*	*48.68± 14.07 *#*	*40.82± 5.26 #*
AST(U/L)	*28.24± 4.84*	*74.10± 3.00 **	*46.32± 6.70 *#*	*54.78± 3.60 *#*	*37.08± 6.52 #@*

*(Values are represented as mean ± SD, *: statistically significant compared to corresponding value in group (I) (P<0.05), #: statistically significant compared to corresponding value in group (II) (P<0.05), $: statistically significant compared to corresponding value in group (III) (P<0.05), @: statistically significant compared to corresponding value in group (IV) (P<0.05)).*

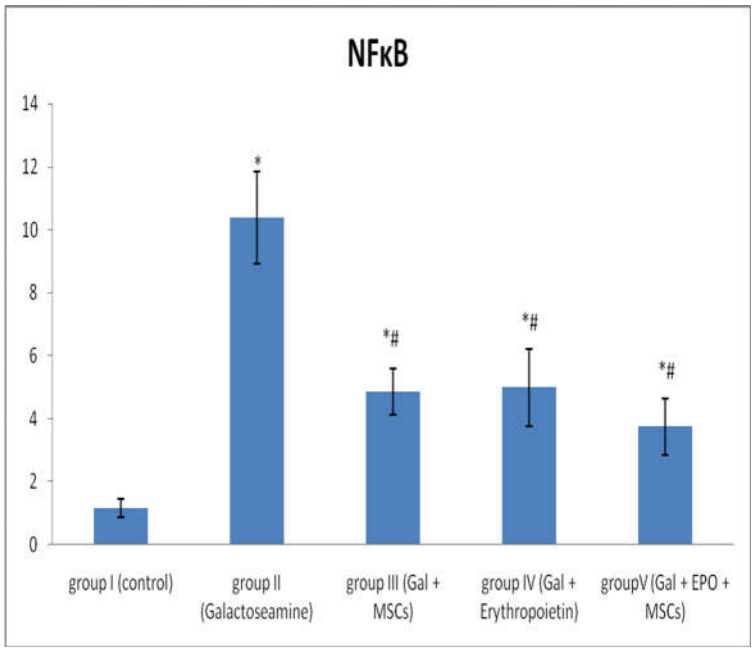

Figure 1(A)

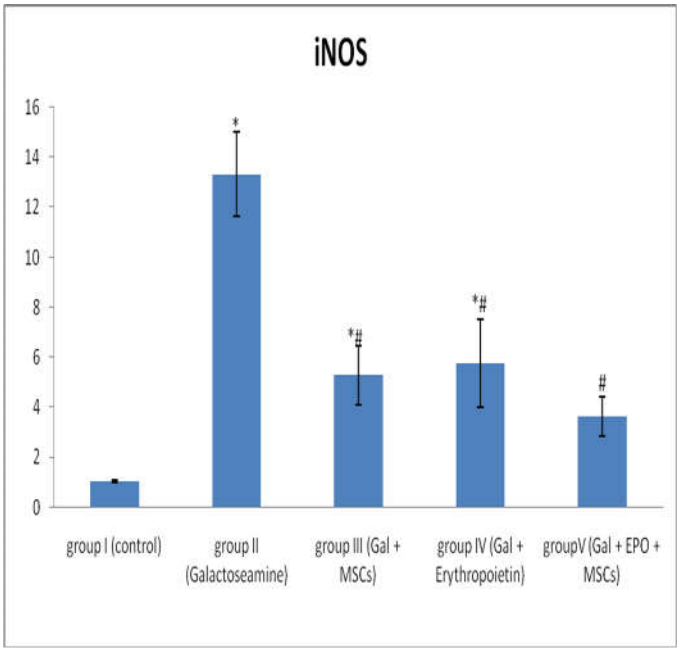

Figure 1(B)

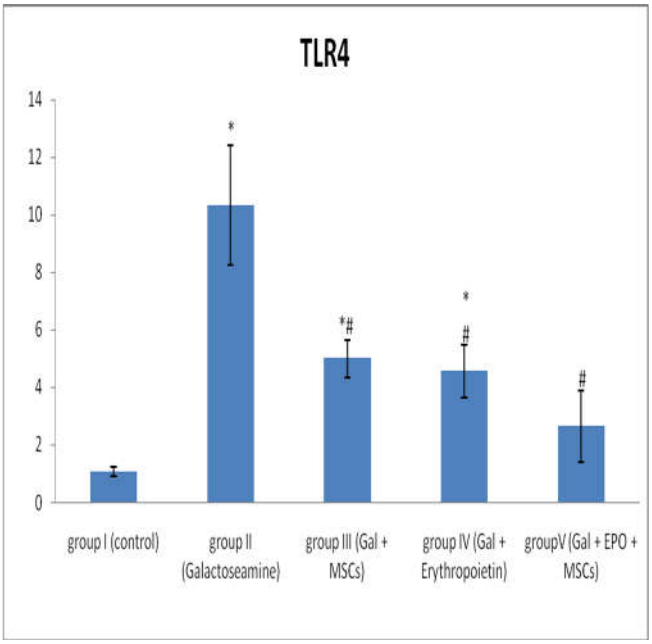

Figure 1(C)

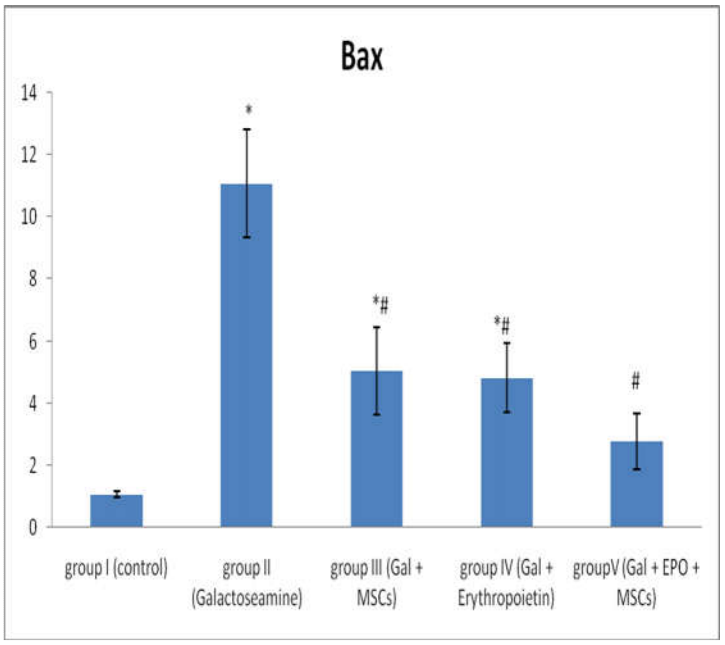

Figure 1(D)

Figure 1: Comparison between the NFκB, iNOS, TLR4 & BAX relative gene expressions in different studied groups (Fig. 1(A), 1(B), 1(C) & 1(D) relatively).

**: statistically significant compared to corresponding value in group (I) (P<0.05), #: statistically significant compared to corresponding value in group (II) (P<0.05).*

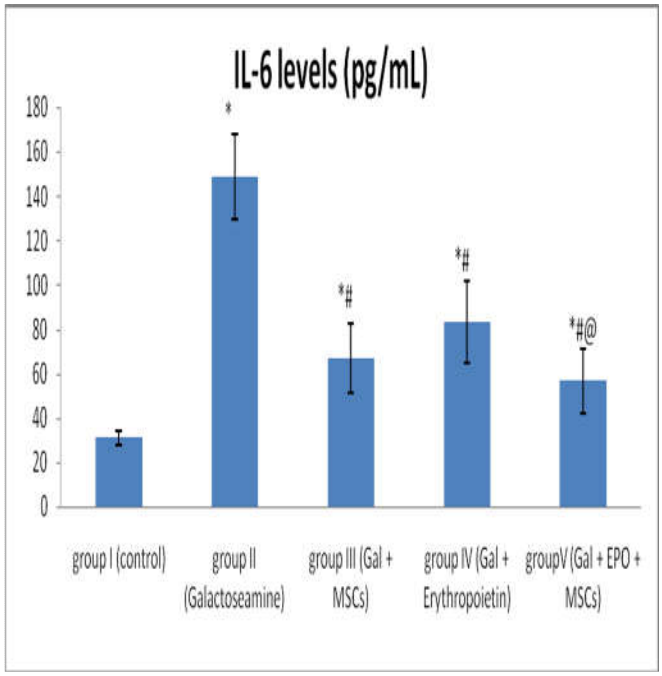

Figure 2 (A)

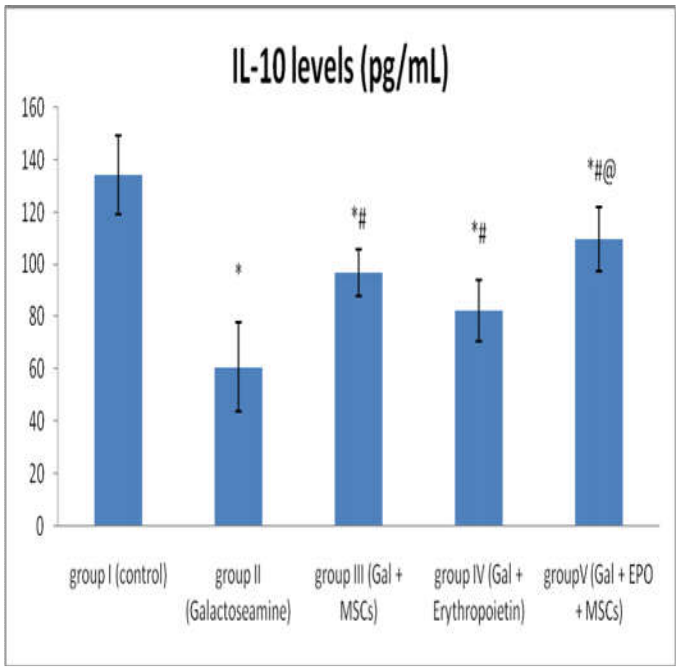

Figure 2 (B)

Figure 2: Comparison between the levels of IL-6 & IL-10 in rat liver of different studied groups (Fig. 2(A) &2(B) relatively).

**: statistically significant compared to corresponding value in group (I) (P<0.05), #: statistically significant compared to corresponding value in group (II) (P<0.05), @: statistically significant compared to corresponding value in group (IV) (P<0.05).*

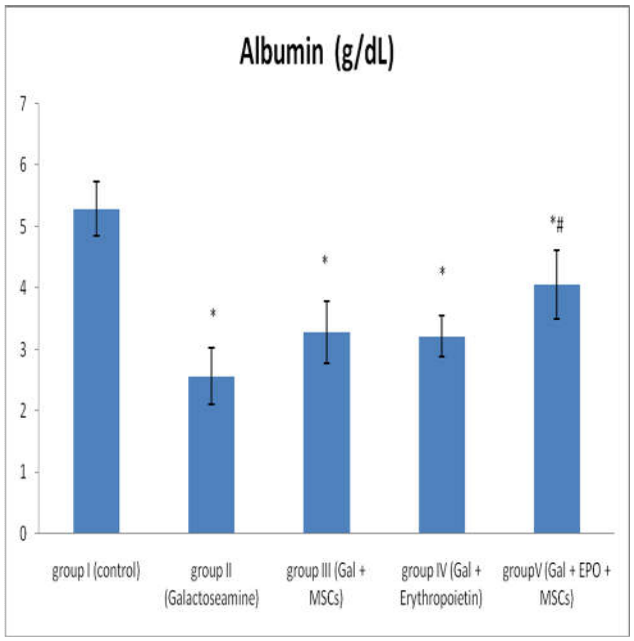

Figure 3 (A)

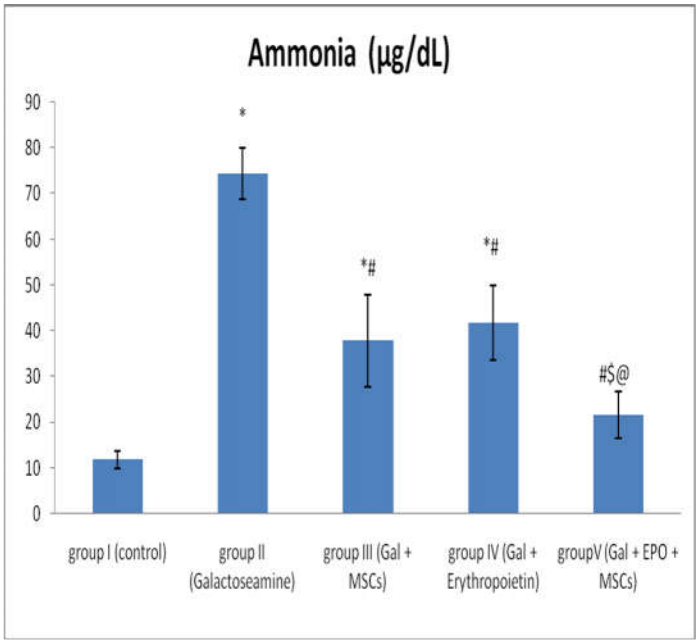

Figure 3 (B)

Figure 3: Comparison between the levels of serum albumin & serum ammonia of different studied groups (Fig. 3(A) & 3(B) relatively).
**: statistically significant compared to corresponding value in group (I) (P<0.05), #: statistically significant compared to corresponding value in group (II) (P<0.05), $: statistically significant compared to corresponding value in group (III) (P<0.05), @: statistically significant compared to corresponding value in group (IV) (P<0.05).*

Histopathological Study

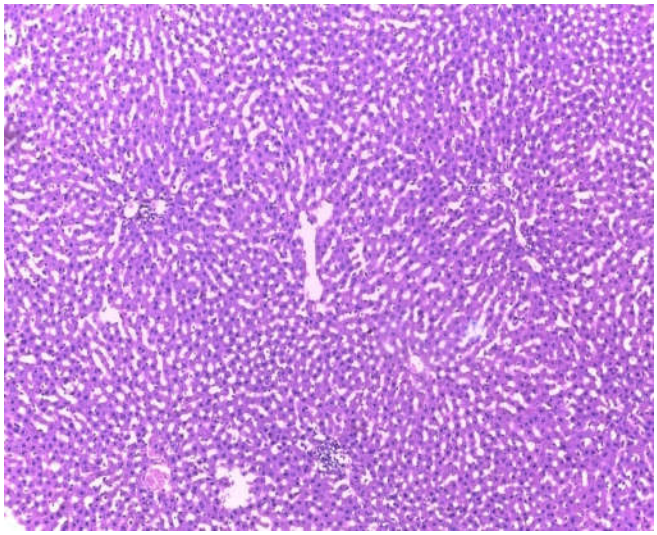

Figure 4(A) (control group)

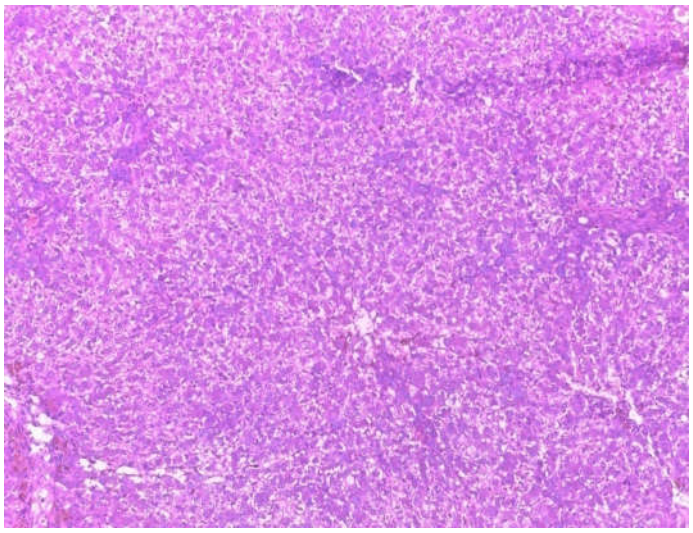

Figure 4(B-i) (GalN group) x100

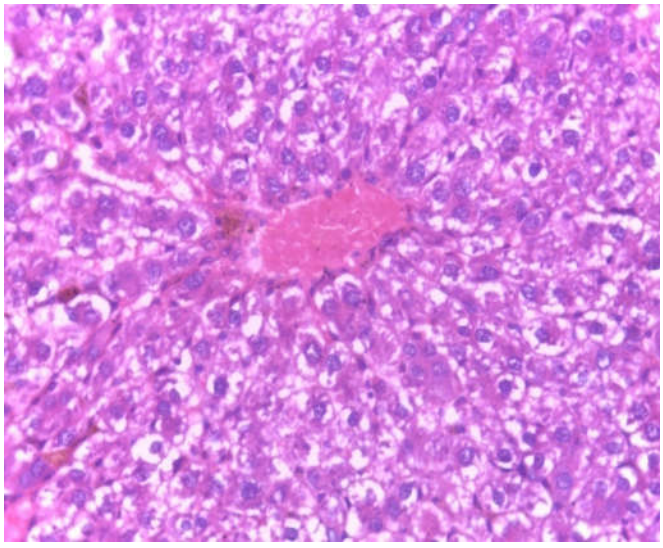

Figure 4(B-ii) (GalN group) x400

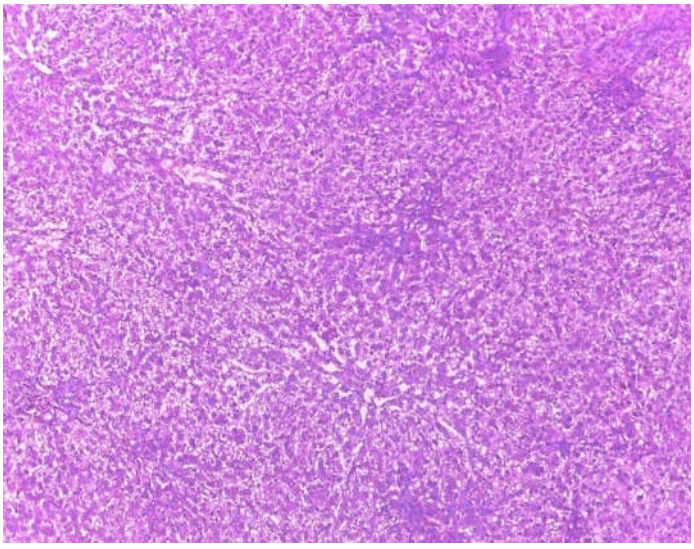

Figure 4(C-i) (GalN +MSCs group)

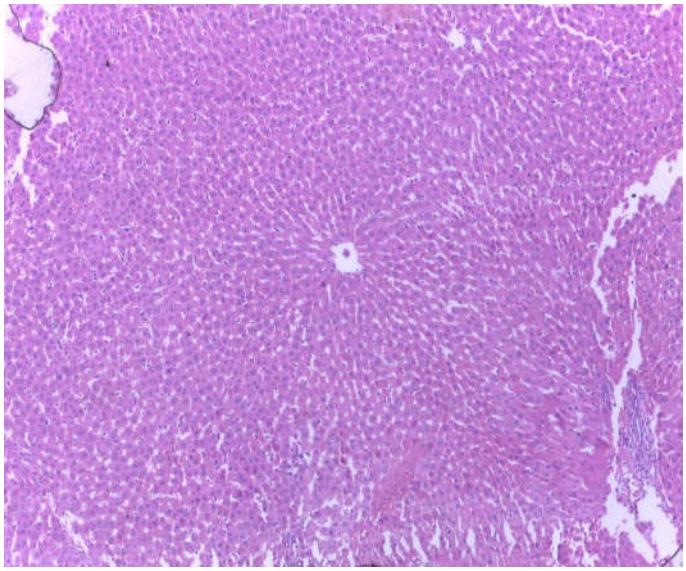

Figure 4(C-ii) (GalN +MSCs group)

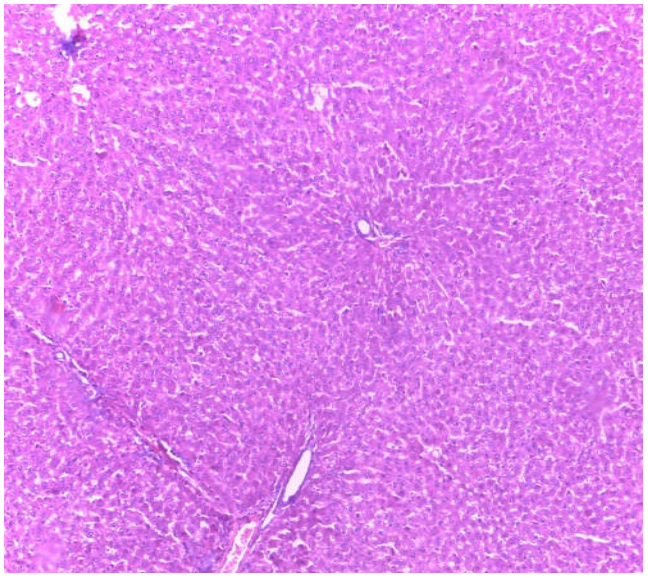

Figure 4(D-i) (GalN+EPO group)

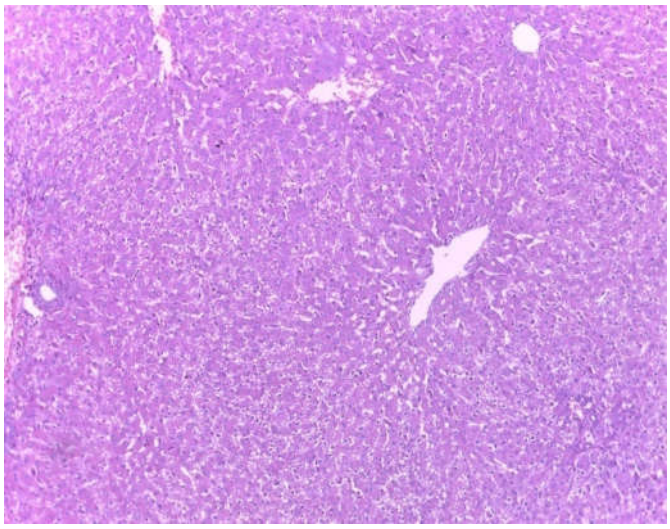

Figure 4(D-ii) (GalN+EPO group)

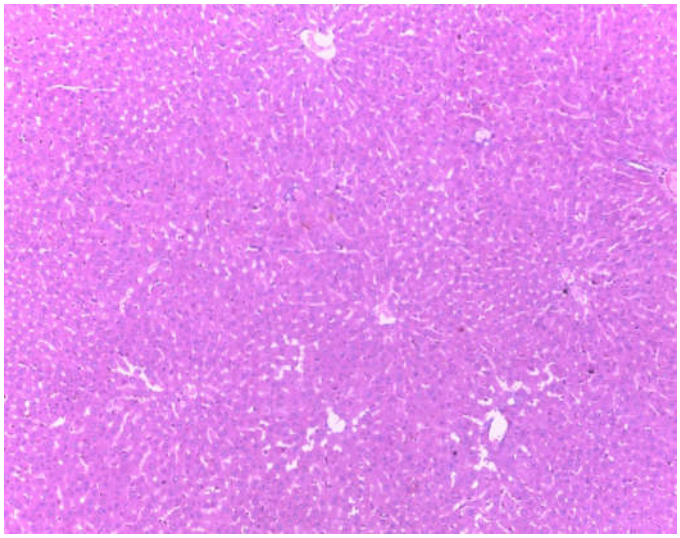

Figure 4(E)(GalN+MSCs+EPO group)

Figure 4: Histopathological pictures of liver tissue of different groups.
- Control group (I) is showing normal lobular hepatic architecture fig.4-A.
-GalN group (II) is showing hepatic focal necrosis and degeneration (vacuolated cytoplasm, large vesicular nuclei, prominent nucleoli, scattered binucleoli and hemorrhage) fig. 4-B (i & ii).
- GalN +MSCs group (III) is showing protective effect of MSCs with a picture of no hepatic focal necrosis and decreased degeneration compared to that seen in GalN-injured animals fig. 4-C (i & ii).
- GalN+EPO group (IV) is showing protective effect of EPO with a picture of no hepatic focal necrosis and decreased degeneration compared to that seen in GalN-injured animals fig. 4-D (i & ii).
- GalN+MSCs+EPO group revealed augmented protective effect of both MSCs & EPO on GalN-injured liver tissue with a picture near normal control fig. 4-E.

Discussion

Galactosamine (GalN) is considered as one of liver intoxicants that induces an intense oxidative stress in hepatocytes [9]. GalN induces liver injury closely resembling viral hepatitis. Administration of GalN disrupts the permeability of the plasma membrane, causing leakage of enzymes from the cell [10].ALT & AST and ammonia significantly increased while albumin significantly decreased in response to GalN injection.

MSCs represent a hopeful gate in protective and treatment strategies through many mechanisms including engraftment property, paracrine secretion activity and transdifferentiation capacity [11]. MSCs-treated group showed a decrease in ALT & AST level and a notable compensation in albumin and ammonia levels compared to GalN-injected group. Like MSCs, EPO showed the same effect on ALT, AST, albumin and ammonia.

MSCs / EPO- treated group showed near normal ALT & AST levels. Also albumin and ammonia levels almost compensated to normal. Based on its functions as an anti-apoptotic, mitogenic, and tissue- protective multifunctional cytokine, many studies suggest that erythropoietin (EPO) may improve liver function and promote liver regeneration [12].

MSCs-treated group and EPO-treated group showed a less severe histopathological picture of hepatocellular necrosis compared to GalN-injected group. A picture close to normal lobular hepatic architecture was observed in MSCs /EPO-treated group (fig-4). MSCs as well as recombinant Epo have been reported to exert mitogenic effects.

The liver injury critically depends on macrophage-derived pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6) and nuclear factor κB (NF-κB). The macrophages of body tissues, especially Kupffer cells of liver, are known to largely contribute to the production of these cytotoxic and inflammatory mediators [13].

GalN induced significant increase in pro inflammatory IL-6 and significant decrease in anti inflammatory interleukin-10 (IL-10). Reversed results were seen with MSCs and EPO injection individually with very impressive results concerning IL-6 & IL-10 were seen in MSCs /EPO-treated group suggesting accumulative effect between MSCs and EPO.

Direct cellular damage and oxidative stress could activate transcription factors including NF-κB which regulate the expression of various inflammatory genes implicated in hepatotoxicity [14] which is very clear in GalN-injected group. It is synchronized with high expression of Toll-Like Receptor-4 (TLR4) which is responsible for induction and

production of reactive oxygen species and expression of pro-inflammatory cytokines such as TNF-α may through activation of NF-κB [15].

The expression of iNOS and Cyclooxtgenase-2 (COX-2) has been shown to be dependent on NF-κB activation [16]. NO production due to cytokine-induced expression of inducible nitric oxide synthase (iNOS) is largely involved in the pathogenesis of inflammatory disorder, including toxin induced liver damage which is already occuring in response to GalN toxicity.

At the level of gene expression, GalN not induces inflammatory genes only but induces the pro-apoptotic gene, BAX which is a cytosolic protein in normal living cells, that can induce apoptosis and quickly translocate to mitochondria at an early stage of the apoptotic process [17].

MSCs and EPO separately protect liver tissues against GalN toxicity at level of gene expression considering NF-κB, TLR4, iNOS and BAX. But the most remarkable protective effect was obtained from combined administration of both MSCs and EPO in the form of MSCs pretreated with EPO. These cells exert anti-apoptotic effect may through increasing the BCL-2 to BAX ratio and inhibiting the activation of caspase-3 partially by paracrine action [18].

EPO expresses tissue protection via inhibition of NF-κB activation. Moreover, EPO impairs the formation of pro-inflammatory factors such as TNF-α, IL-6, IL12/IL-23 subunits and NO via inducible NO synthase (iNOS) by macrophages [19]. EPO observed anti-apoptotic effects could partial explained by the noted effects on the antioxidant enzyme Superoxide Dismutase (SOD) and glutathione which were significantly increased after erythropoietin treatment [20].

Conclusion

Administration of MSCs and EPO may have hepatoprotective effect on GalN-induced hepatotoxicty, particularly the MSCs pretreated with EPO. The mechanism of this hepatoprotective effect may be through anti-inflammatory, anti-apoptotic and immunomodulation actions of MSCs and EPO.

الملخص العربى

ينشأ الفشل الكبدي الحاد من الإصابة الكبدية المفاجئة والحادة نتيجة لأسباب عدة. ويرتبط ذلك مع ارتفاع معدلات الوفيات وزيادة التكلفة الاقتصادية. ويعد السبب الأكثر شيوعا لزارعة الكبد في العديد من البلدان. يعتبر الجالاكتوزامين احد السموم الكبدية المعروفة وغالبا ما يستخدم لإستحداث الفشل الكبدي الحاد فى حيوانات التجارب. من خلال آليات مختلفة، يؤثر الجالاكتوزامين على أغشية الخلايا والعضيات و ايضا يؤثرعلى تكوين البروتينات والأحماض النووية.

يمتلك الإرثروبويتين – وهو هرمون واقى للأنسجة- العديد من الوظائف أكثر مما كان يعتقد سابقا. وذلك كونه يقوم بوقاية الأنسجة من خلال مكافحة موت الخلايا المبرمج، ونشاطة كمضاد للأكسدة وايضا مكافحة الالتهابات. توفر الخلايا الجذعية الوسيطة - بقدرتها الفائقة على التجدد والتمايز- فرص واعدة لعلاج العديد من

الأمراض بما في ذلك الفشل الكبدي الحاد. و من أهم آليات المفترضة لعمل الخلايا الجذعية الوسيطة؛ التميز لخلايا فعالة و الإنصهار داخل خلايا اخرى وعملها كغدة ذات افراز مجاور ونقل الميتوكوندريا وايضا امكانية ارسال حويصلات تحمل الأحماض النووية.

تم عمل هذه الدراسة داخل جسم حيوانات التجارب وقد شملت مائة فأر ذكر متقاربين فى الوزن. تم تقسيم الحيوانات إلى 5 مجموعات تشتمل كل منها على 20 فأر:

المجموعة 1: فئران اصحاء ، المجموعة 2: تم حقنهم جالاكتوزامين (650 مج / كج) لإستحداث فشل كبدى حاد ، المجموعة 3: تم حقنهم با لخلايا الجذعية الوسيطة (واحد مليون خلية / فأر) بعد حقنهم جالاكتوزامين ، المجموعة 4: تم حقنهم الإرثروبويتين (12 وحدة دولية / كغ) بعد حقنهم جالاكتوزامين والمجموعة 5: تم حقنهم با لخلايا الجذعية الوسيطة معالجة بالإرثروبويتين بعد حقنهم جالاكتوزامين .

في الوقت المخطط (7 أيام)، تم جمع الدم وأنسجة الكبد.استخدمت أنسجة الكبد في الفحوصات التالية:

- النشاط الجيني لكل من الجينات الأتية NFκB، iNOS، TLR4 وBAX باستخدام تقنية تفاعل البلمرة المتسلسل الكمي اللحظي .

- مستوى الإنترلوكين 6 و الإنترلوكين 10 باستخدام المقايسة المناعية المرتبط بالإنزيم (اليزا).

- تقييم الحالة المرضية لنسيج الكبد.

وقد تم تقييم وظائف الكبد عن طريق قياس الزلال، والأمونيا،و كذلك مستوى انزيمات الكبد (ALT& AST) في مصل الدم.

أظهرت نتائج الدراسة عن التأثير السام للجالاكتوزامين حيث انه يقلل مستوى الزلال و الإنترلوكين 10 في حين يزيد مستوى انزيمات الكبد (ALT& AST)، والأمونيا و الإنترلوكين 6 ويزيد أيضا النشاط الجيني لكل من NFκB، iNOS، TLR4 وBAX. لقد أوضحت النتائج ان كل من الإرثروبويتين و الخلايا الجذعية الوسيطة له دور فى حماية الكبد ضد تأثير سمية الجالاكتوزامين بدرجة متقاربة. حيث أنهما وبشكل منفصل يعملا على زيادة مستوى الزلال و الإنترلوكين 10 التى انخفضت نتيجة سمية الجالاكتوزامين. واديا الى انخفاض ملحوظ فى مستوى انزيمات الكبد (ALT& AST)، والأمونيا، و الإنترلوكين 6 المرتفعة من قبل الجالاكتوزامين. أيضا، قام كل من الإرثروبويتين و الخلايا الجذعية الوسيطة بخفض نشاط جينات NFκB، iNOS، TLR4 وBAX التي أثارتها سمية الجالاكتوزامين.

أظهرت النتائج ايضا ان استخدام الإرثروبويتين و الخلايا الجذعية الوسيطة مجتمعين فى صورة خلايا جذعية وسيطة معالجة بالإرثروبويتين ادى الى زيادة درجة الحماية عن استخدام كل على حدى. حيث أدى إلى تحسن مستوى مختلف الفحوصات (الزلال، والأمونيا، انزيمات الكبد (ALT، AST)، الإنترلوكين 6، الإنترلوكين 10، NFκB، iNOS، TLR4 وBAX) المضطربة بسبب الجالاكتوزامين لدرجة مقاربة لمستواهم فى الفئران الأصحاء. و أدى ايضا إلى تحسن الصورة المرضية لنسيج الكبد بسبب الجالاكتوزامين أقرب إلى الصورة الطبيعية لنسيج الكبد.

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