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Hepatoprotective Effects of Mesenchymal Stem Cells and Erythropoietin on D-galactosamine Induced Hepatic Failure in Rats

Reda Hassan[1 *], Samir A. Zaahkouk[1,] Mansour A. [2] * and Sawsan El-Shamy [3]

1-Zoology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt
2-Pharmacology Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt
3-Biology Department, Basic Science Center, Misr University for Science and Technology, Cairo, Egypt.
* Corresponding author

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Background
Mesenchymal stem cells (MSCs), with their remarkable capacity for self-renewal and differentiation, provide opportunities for treating many diseases, including acute liver failure. Erythropoietin (EPO) demonstrates both hematopoietic functions and anti-inflammatory properties. Its role in tissue protection has been validated across a spectrum of injuries, including those affecting the kidneys, liver, myocardium, and brain. Experimentally, acute liver injury can be induced by the well-known and often-used hepatotoxic galactosamine (GalN).
Materials and Methods: Sixty-six adult male albino rats were divided into eleven groups, each consisting of 6 rats. A range of biochemical and molecular parameters were evaluated in the liver, including the expression levels of IL-6 and IL-10, NFκB activity, BAX and iNOS gene expression, alongside histological analysis using Masson's staining technique.
Results: The administration of MSCs and EPO mitigated the toxic impact of GalN by normalizing the mean expression levels of NOS, NFκB, BAX, TLR4, IL-6, and IL-10 genes. Notably, the concurrent use of MSCs and EPO provided enhanced protective effects against GalN-induced toxicity.
Key words: Stem cells - Erythropoietin - Hepatic injury - Galactosamine (GalN)**.**

INTRODUCTION

Acute liver failure (ALF) can result in extensive hepatocyte necrosis and profound hepatic impairment. This cascade rapidly progresses to hepatic encephalopathy and multiorgan dysfunction, which, if left untreated, often culminates in the swift demise of the patient, barring the intervention of a liver transplant **(Mounz, 2014).** The liver possesses an exceptional regenerative capacity, which may enable full recovery even in cases of substantial hepatic injury and significant loss of liver mass. Thus, developing interventions that can postpone the mortality associated with ALF would be of considerable clinical significance.

Therefore, there is a critical need for innovative therapeutic techniques for ALF. In recent times, stem cell transplantation has offered an alternative strategy for patients to prolong their lives (Zhang et al., 2022).

Nevertheless, liver transplantation is presently constrained by prohibitive costs, significant risks of immune rejection, and a critical shortage of donor organs. Recently, stem cell transplantation has emerged as the preferred alternative to mitigate dependency on liver transplantation (Hu 2020).

Stem cells are multipotent entities with the capacity for proliferation, self-renewal, and differentiation into diverse somatic cell types under in vitro conditions. These cells have garnered significant interest for their potential to restore tissue functionality in vivo with minimal immune rejection. MSCs can be derived from a variety of sources, including bone marrow, adipose tissue, amniotic membrane, placenta, and other tissues (Chen 2019).

Galactosamine (GalN) is a widely recognized and frequently employed hepatotoxic agent in the study of liver regeneration models. In experimental animal models, administration of a single dose of GalN

triggers acute hepatitis, while successive doses lead to the onset of chronic liver failure **(Wang *et al*., 2013)**.

The hepatotoxicity induced by GalN arises from the depletion of uridine diphosphate (UDP)-glucose and UDP-galactose, critical components for RNA and protein synthesis, coupled with a reduction in intracellular calcium levels. This dual deficit leads to compromised cell membrane integrity, organelle dysfunction, and a disruption in the synthesis of proteins and nucleic acids (**Keppler *et al*., 1994)**.

The intrinsic ability of stem cells to self-renew and differentiate into distinct organ-specific cell types equips tissues with the remarkable capability to regenerate and preserve functionality after diverse forms of injury (**Bochon *et al*.,** 2019**)**.

Beyond their well-established differentiation into osteoblasts and adipocytes, bone marrow-derived mesenchymal stem cells (BM-MSCs) possess the potential to diversify into various other mesenchymal lineages. Extensive in vitro and in vivo studies have thoroughly explored the ability of human BM-MSCs (hBM-MSCs) to undergo differentiation into hepatocyte-like cells. Additionally, these cells exhibit the ability to undergo prolonged culture expansion without a notable decline in their differentiation potential. Several research groups have initiated clinical trials involving the transplantation of autologous bone marrow cells in patients suffering from cirrhosis or chronic liver fibrosis. Nevertheless, the application of hBM-MSCs for the treatment of fulminant hepatic failure (FHF) remains largely unexplored in both animal models and human patients, despite the significant clinical relevance such studies could hold **(Li *et al*., 2012)**.

Erythropoietin (EPO) serves as a crucial pharmacological agent for the management of anemia, chronic kidney disease, and various other medical

conditions **(Eschbach *et al.*, 1987)**. EPO exerts a range of protective effects, demonstrating antiapoptotic, antioxidant, and anti-inflammatory properties **(Yang, 2014).**

EPO, recognized for its tissue-protective and regenerative potential across various organs, including the heart, brain, spinal cord, and kidneys, has demonstrated the ability to enhance MSC recruitment and promote angiogenesis **(Chen,2014)**. The erythropoietin receptor, present on the surface of BMSCs, has been implicated in augmenting the proliferation of BMSCs in response to acute injury scenarios (**Liu *et al.*, 2013**).

For successful application, an adequate number of MSCs must effectively home to the target tissues post-administration. However, a significant challenge in MSC therapy is the inefficiency of MSC homing following systemic administration (Yuan et al., 2022).

During fetal development, EPO is predominantly synthesized by the liver. Roughly 80% of EPO production is primarily linked to the kidneys, which serve as the dominant source following birth. Additionally, the presence of EPO mRNA in the spleen, bone marrow, lungs, and brain suggest that these organs may contribute to limited EPO synthesis **(Jelkmann, 2013)**.

The imbalance between pro- and anti-inflammatory cytokines is the defining feature of the pathogenesis of acute liver injury (ALI), which is linked to the production of pro-inflammatory cytokines (IL-6, IL-1b, and TNF-a) **(Li et al., 2017).** Nuclear factor κB (NF-κB), iNOS, and cyclooxygenase-2 (COX-2) are essential mediators of inflammation and cytotoxicity. The macrophages residing within body tissues, particularly the Kupffer cells of the liver, play a significant role in the synthesis of these inflammatory agents **(Liong *et al.*, 2012)**.

The activation of NF-κB within macrophages triggers the release of pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-1, IL-6, and IL-12

(Zhan *et al*., 2014). The expression of iNOS and COX-2 has been shown to be dependent on NF-κB activation **(Kang *et al*., 2011).** NF-κB, along with the genes under its regulatory control, plays a pivotal role in immune and stress responses, influencing critical cellular processes including apoptosis, proliferation, differentiation, and development **(Oeckinghaus and Ghosh, 2009**).

The generation of NO through the iNOS-catalyzed oxidative deamination of L-arginine, triggered by pro-inflammatory cytokines or endotoxins, can provoke harmful cellular reactions, leading to inflammation and sepsis **(Bultinck *et al*., 2006)**.

Regeneration and fibrosis are interconnected processes initiated by injury, with the subsequent pathway diverging based on the extent and damage duration. This complex cascade involves the coordinated interaction among mesenchymal, epithelial, endothelial, and immune cells. During liver injury, Kupffer cells recognize dead hepatocytes or foreign antigens, leading to the activation of recruited monocytes into macrophages. The Smad signaling pathway enhances the transcription of collagen types I and III by stimulating the production of TGF-β in these macrophages. Additionally, these cells release pro-inflammatory cytokines, including IL-1β and TNF-α, which facilitate hepatic collagen deposition and sustain the viability of activated hepatic stellate cells, characterized by α-SMA positivity **(Pradere *et al*., 2013)**

Cheng-Maw *et al*. (2020) showed extensive hepatocyte necrosis in the liver tissue after one day of GalN administration. While progressive restoration with decreased inflammatory cell infiltration was indicated in the liver parenchyma after cotransplantation with MSCs.

The potential benefits of immunosuppressive agents in promoting liver regeneration, both pre- and post-liver transplantation, are indicated by the

infiltration of T cells, B cells, and monocytes, which is a marker of chronic liver injury caused by inflammation (Manousou et al., 2010). MSCs possess immunomodulatory properties that can similarly aid this process by inhibiting T cells through the release of various factors, including nitric oxide, indoleamine 2,3-dioxygenase, prostaglandin E (PGE)-2, IL-10, IL-6, and human leukocyte antigen G. The proliferation and functionality of a variety of immune cells are influenced by these factors. The synergistic action of cytokines, including interferon-γ, IL-1α, and TNF-α, is the catalyst for the immunosuppressive efficacy of MSCs. Moreover, MSCs can inhibit B cell activation, leading to reduced immunoglobulin production (Zhang et al., 2008). Co-culture with MSCs has been linked to a marked decrease in the expression of chemokine receptors CXCR4, CXCR7, and CXCR5 (Glennie et al., 2005).

Materials and method:

1- Experimental animals:

Sixty-six male rats from the inbred strain (Cux1: HEL1), with uniform weights ranging from 180 to 220 grams, were bred within the Experimental Animal Unit at the Faculty of Sciences, Al-Azhar University. The research adhered rigorously to the established protocols set forth by the Institutional Animal Care and Use Committee and obtained formal approval from the Institutional Review Board. The rats were sustained on a semi-purified diet comprising the following components per kilogram: 200 grams of casein, 555 grams of sucrose, 100 grams of cellulose, 100 grams of fat blends, 35 grams of vitamin mixes, and 35 grams of mineral mixes.

Experimental design: Animals were divided into 11 groups; each group consists of 6 rats.

Gr. I: **The** control group.

Gr. II: To cause acute liver toxicity, rats received a single injection of galactosamine (GalN) (1700 mg/kg IP) dissolved in saline **(Bigoniya *et al.*, 2009)**.

Gr. III: Two hours after the GalN treatment (1700 mg/kg IP), rats received a single infusion of MSCs (one million cells/rat IP). **Volarevic *et al. (*2014)**

Gr. IV: Within 24 hours, rats received a single infusion of MSCs (one million cells/rat IP), following an administration of GalN (1700 mg/kg IP). Lipsic *et al*. (2008)

Gr. V: Rats received a single intraperitoneal injection of MSCs (one million cells per rat) 48 hours following the administration of GalN (1700 mg/kg IP).

Gr. VII: Immediately following the injection of GalN, rats will receive a single infusion of MSCs (one million cells/rat IP) 72 hours later. (1700 mg/kg IP).

Gr. VIII: Rats were administered a single intraperitoneal injection of the MSC fraction (one million cells per rat) daily for four consecutive days, commencing immediately after the GalN injection (1700 mg/kg IP).

Gr. IX: Rats were injected with EPO (12 IU/kg IP) once following GalN injection. (1700 mg/kg IP) as modified from **Lipsic et al. (2008).**

Gr. X: Rats were administered EPO (12 IU/kg IP) and MSCs (one million cells/rat) immediately following GalN injection (1700mg/kg IP).

Gr. XI: Rats were administered once with EPO (12 IU/kg IP) and MSC fraction for 4 days immediately after GalN injection (1700mg/kg)

Gr. 11: The rats were injected with one million MSCs immediately following the GalN injection (1700 mg/kg IP) in the portal vein. The method explained by Samir et al. (2024)

Immediately after the GalN injection (30 days). Rats in all groups had their retroorbital veins used to draw venous blood, and the animals were killed by dislocating their cervical vertebrae. Liver tissue was excised and subsequently divided into two distinct portions for analysis. The following assessments were conducted: 1) Quantification of IL-6 and IL-10 levels

within the tissue using ELISA, alongside the evaluation of gene expression for NFκB, iNOS, TLR4, and BAX. Gene expression levels were estimated via quantitative real-time polymerase chain reaction (qRT-PCR). 2: Histopathological studies: the liver was quickly removed, fixed in paraformaldehyde at 4°C, and paraffin bees wax tissue blocks were prepared for sectioning at 4 microns of thickness by slidge microtome and stained with Masson stain for general histological structure (Suvarna *et al*., 2018).

Estimation of NFκb, iNos, and BAX gene expression

Total cellular RNA was isolated using **the RNeasy mini kit** and reverse transcripted using the **Quantiscript RTPCR kit.** qPCR amplification and melting curve analysis were performed using Quiagen on the Applied Biosystems (Kaser **Al Eini Medical School*)***. detection system with software version 3.1 ***(StepOneTM, USA)***. The method was used to determine relative mRNA expression levels after normalization of GAPDH as a qPCR assay with the primer sets optimized at the annealing temperature (25–60 °C.).

Table 1: The oligonucleotide primer sequence of the studied genes (in vivo).

Parameters	**Primer sequence**
NFκB	**Forward primer:** 5′- GCTTACGGTGGGATTGCATT - 3′ **Reverse primer:** 5′- TTATGGTGCCATGGGTGATG - 3′
iNOS	**Forward primer:** 5′- GGGCCACCT TTATGTTTGTG - 3′ **Reverse primer:** 5′- CCTCAACCTGCTCCTCACTC -3′
BAX	**Forward primer:** 5′- GTTGCCCTCTTCTACTTTG - 3′ **Reverse primer:** 5′- AGCCACCCTGGTCTTG - 3′
GAPDH	**Forward primer:** 5′ - TGCTGGTGCTGAGTATGTCG - 3′ **Reverse primer:** 5′ - TTGAGAGCAATGCCAGCC - 3′

4 -In vivo study

- **Isolation, propagation, labeling and identification of bone marrow-derived MSCs from rats:**

Following cervical dislocation, bone marrow was harvested from rodents post-sacrifice. The isolated cells were cultured under sterile conditions, and upon the formation of substantial colonies, the cultures were thoroughly rinsed twice with phosphate-buffered saline. Subsequently, the cells were detached using a trypsin solution containing 1mM EDTA. The femur and tibia of the rodents were carefully excised during the procedure. After centrifugation, cells were re-suspended with serum-supplemented medium and incubated **(Alhadlaq and Mao, 2004)**. On day 14, the adherent colonies of cells were trypsinized, and counted. MSCs cells were harvested during the 4th passage and were labeled with PKH26 fluorescent linker dye. Undifferentiated MSCs were injected intravenously into the rat tail vein. The morphology of the cultures was studied by optic and transmission electron microscopy. The cells underwent analysis via flow cytometry. Additionally, the liver tissue was scrutinized under a fluorescent microscope to confirm cell homing and trace the presence of the injected cells within the liver tissue. All the above steps were performed as explained by **Samir et al. (2024).**

5. **In vitro study**

Bone marrow-derived mesenchymal stem cells were isolated from rats following the procedure outlined by Liu et al. (2011-b). The cells were cultured, and after 48 hours, they were harvested and divided into two equal fractions, with each fraction corresponding to a distinct group. Each group was further subdivided into six subfractions. The first group received treatment with 10 IU/mL EPO for five consecutive days ***(Liu et al., 2011-b). The second*** group served as a control group.

Cell proliferation MTT assay. Then cells were harvested, total RNA extraction was performed, and RNA was reverse transcripted for caspase-3 and BAX/BCL-2 ratios. Gene expression estimation using RT-qPCR and the primers represented in Table 2 . Then, cell proliferation was estimated.

Table (2): ***Table 2: The oligonucleotide primer sequence of the studied genes (in vitro).***

	Primer sequence	
Caspase -3	**Forward primer :**	5′- CTGGACTGCGGTATTGAGAC - 3′
	Reverse primer:	5′- CCGGGTGCGGTAGAGTAAGC - 3′
BAX	**Forward primer :**	5′- GTTGCCCTCTTCTACTTTG - 3′
	Reverse primer:	5′- AGCCACCCTGGTCTTG - 3′
BCL-2	**Forward primer :**	5′- CGGGAGAACAGGGTATGA - 3′
	Reverse primer:	5′- CAGGCTGGAAGGAGAAGAT - 3′
GAPDH	**Forward primer :**	5′ - TGCTGGTGCTGAGTATGTCG - 3′
	Reverse primer:	5′ - TTGAGAGCAATGCCAGCC - 3′

2. Cell proliferation assay

Estimation of cell viability and proliferation using the MTT assay.

Cell viability and proliferation control and EPO-treated cells were evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide (MTT) assay according to manufacturer instructions.

Statistical analysis

All data are represented as the mean ± standard error of the mean.

The results were subjected to oneway analysis of variance (ANOVA) sub sequently followed by Tukey's multiple comparison tests. A P-value of less than 0.05 was deemed indicative of statistical significance.

Results

In vitro

1- MSCs Isolation, propagation, identification and labeling MSCs in culture.

MSCs isolation from bone marrow was carried out under the previously mentioned conditions and maintained for 14 days before subculture. The cells were observed under the inverted microscope at one week and two weeks to assure the morphology of the cells **(Fig. 1A and B).** The presence of MSCs in the liver tissue was confirmed following their labeling with PKH26 fluorescent dye, demonstrating that these cells successfully migrated and localized to the liver.

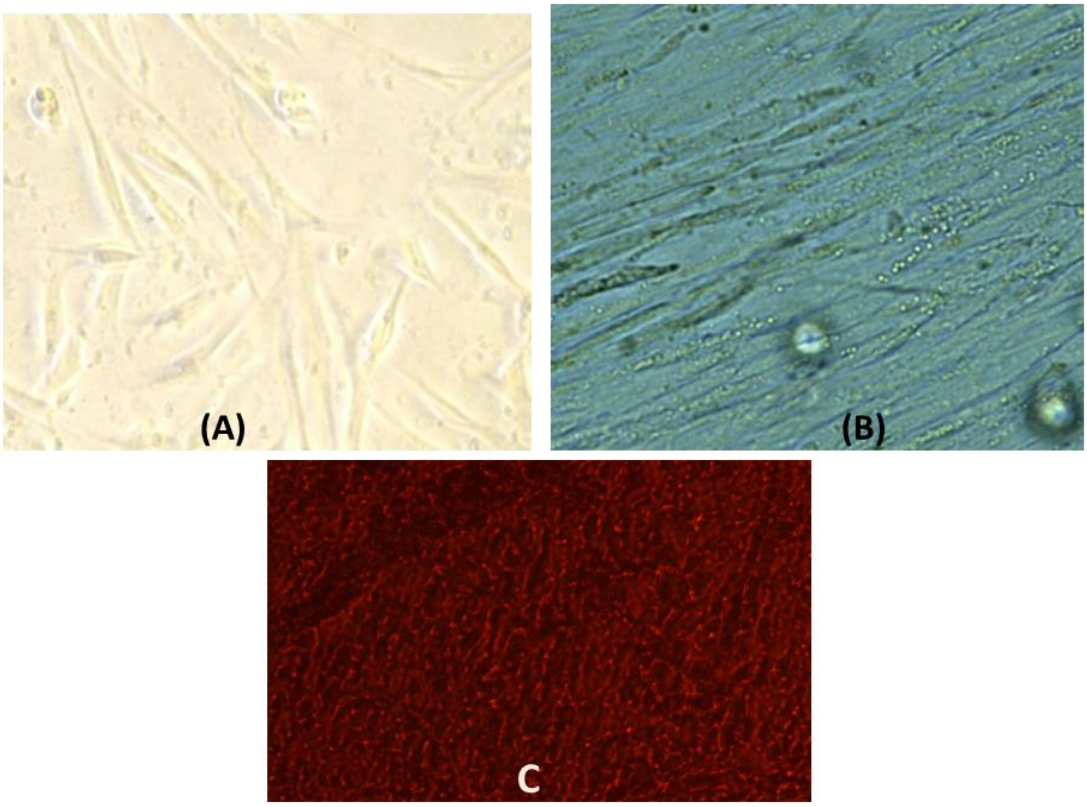


Fig. 1 (A): Spindle-shaped MSCs formed after a week in culture. ***(B)*** Two weeks after culture, MSC morphology. ***(C)*** MSCs are marked using the PKH26 dye. The liver tissue included MSCs stained with PKH26 fluorescent dye, indicating that the cells had migrated into the liver tissue. (**As shown by Samir et al., 2024**)

Analysis of MSCs based on cell surface marker expression:

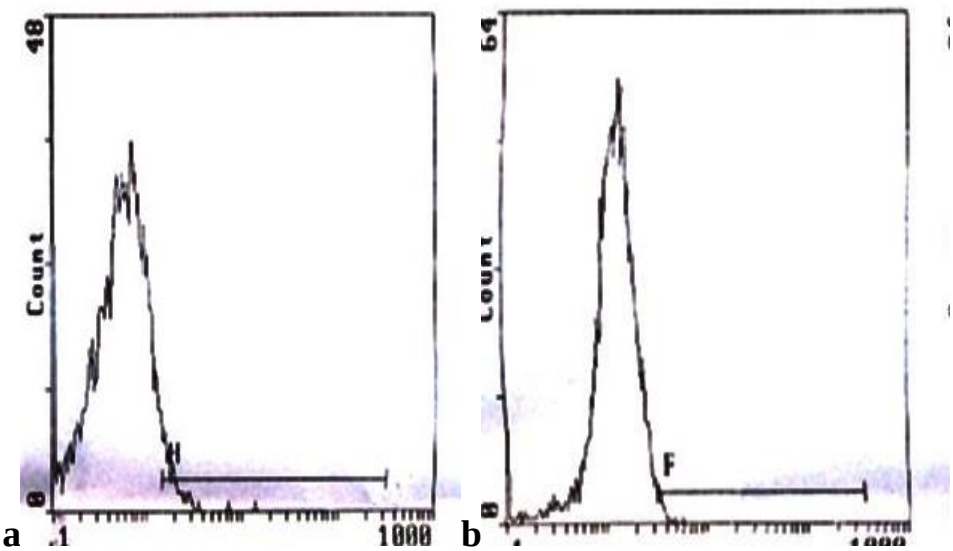


Fig. 2: Flow cytometric analyses (2a) Cells were negative for CD34 (2b) positive for CD29. As indicated by Samir et al. (2024).

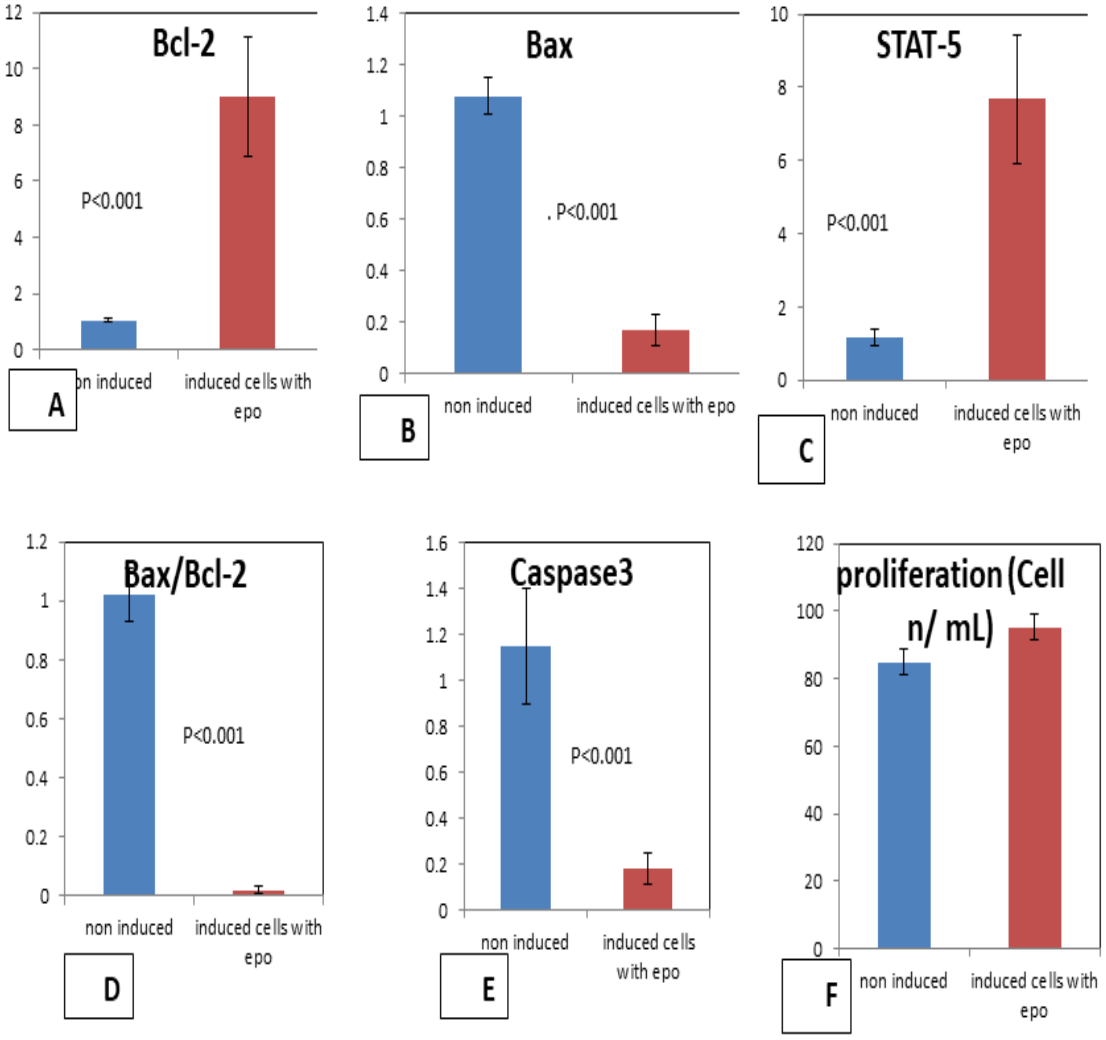


Fig. 3(A, B, C, D, E and F): qPCR analysis in bone marrow-driven MSC cells for gene expression post-EPO treatment showed upregulation of Bcl-2 gene expression (**fig. 3A**), downregulation of Bax **(fig. 3B)**, upregulation of STAT-5 (fig**. 3C**), downregulation of Bax/Bcl-2 (**fig. 3D),** and Caspase-3 (**fig. 3E**) gene expressions. The ΔΔ CT method was used to estimate the fold change represented by Rq. Rq = 1 represents the control, and the standard error is represented by the error bar. Proliferation activity of the control and EPO-treated MSC cells was cultured for 48 h, and the proliferation was assessed by MTT assay as described before (**fig. 3F**). Highly significant. ***P<0.001.

The current study showed that the IL - 6, BAX, TLR4, INOS, and **NFκB** levels were significantly upregulated (*; *P <0.01). While significant downregulation of IL - 10* in the group injected with GaIN compared to the control group (Figure 4) this refer to the high liver injury. However, comparing the MSC and/or EPO groups to the GalN group (**group II)**, we discovered that these treatments, though given at different times, somewhat attenuated and ameliorated the levels of these parameters.

The group **X** showed the greatest recovery after receiving EPO + MSC fractions for four days.Also, according to the findings. It was discovered that these gene levels significantly improved after receiving a fractionated MSC treatment for four days (250 cells/day).

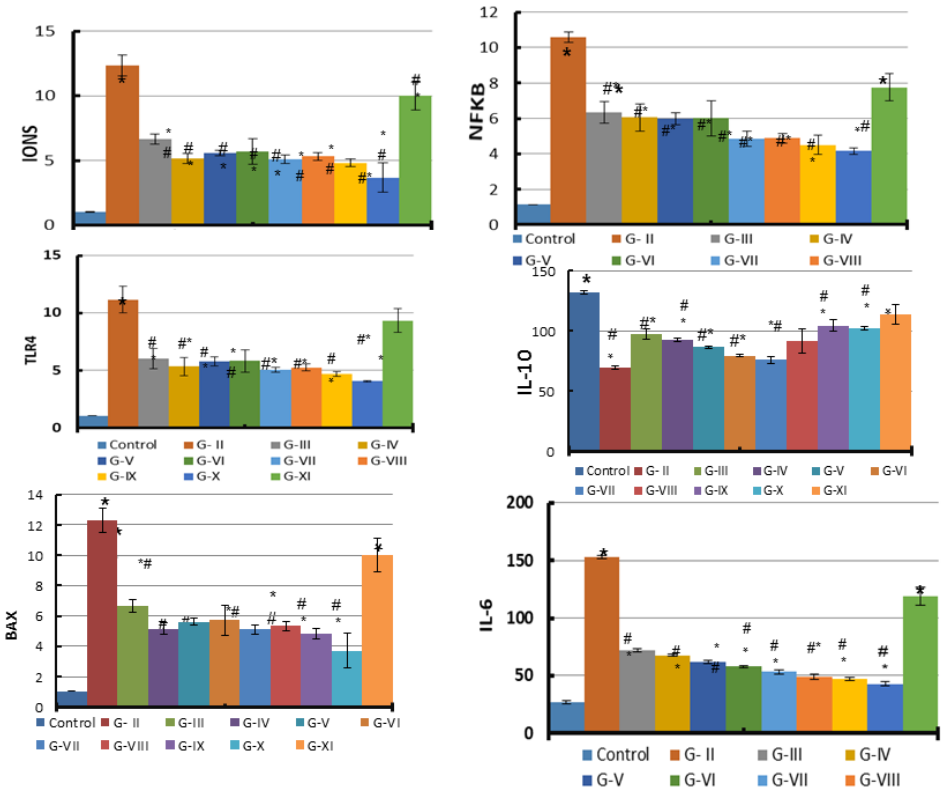
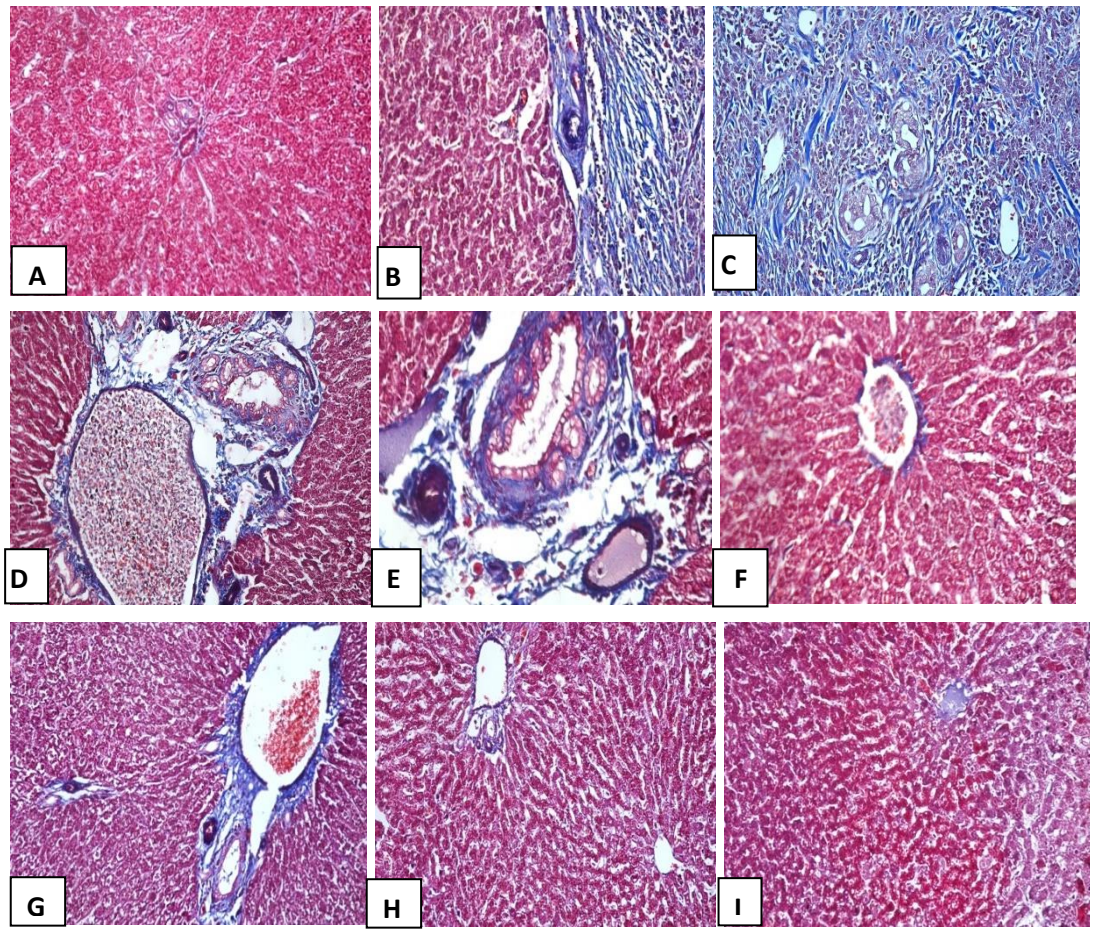


Figure 4: Comparison between the relative gene expression levels in the different studied groups. *: statistically significant compared to corresponding value in group I. ≠: significant in comparison with gr. 2.

Histopathological study

The liver tissue was stained with Masson stain to assess the effect of MSCs and/or EPO on liver damage induced by GalN injection. The control group showed a negative Masson's stain, indicating the absence of hepatic fibrosis (**Fig. 5A).** While the histological examination of the liver tissues showed that the GalN injection showed multi-focal areas of markedly positive bluish Masson's stain, indicating severe hepatic fibrosis (f) (**Figs. 5B and C)**. Administration of MSCs after **2** hours post-GalN injection showed positive bluish Masson's stain in the portal area, indicating portal area fibrosis (arrow), hepatic parenchyma showing no fibrosis, and moderate (50%) regression of the degeneration; note the apparently normal hepatocytes with normal cytoplasm and a centrally located nucleus (arrows) (**Fig. 5D**). While, after 24 hours post-GalN injection showed a

positive bluish Masson's stain in the portal area, indicating portal area fibrosis (arrow), with the hepatic parenchyma showing no fibrosis (**Fig. 5E**). rats administered with MSCs after 48 hours post-GalN injection showed negative Masson's stain, indicating the absence of hepatic fibrosis (**Fig. 5F)**. Examination of the liver tissue of rats administered with MSCs after 72 hours post-GalN injection showed negative Masson's stain, indicating absence of hepatic fibrosis (**Fig. 5G).** Examination of the liver tissue of rats administered with MSC fraction for 4 days post-GalN injection showed negative Masson's stain, indicating absence of hepatic fibrosis (Fig. 5H). Administration of EPO 12 IU/kg post-GalN injection showed negative Masson's stain, indicating the absence of hepatic fibrosis (Fig. 5I**)**. EPO 12 IU/kg IP plus MSCs showed negative Masson's stain, indicating the absence of hepatic fibrosis (Fig. 5J). EPO (12 IU/kg IP + MSCs fraction) showed a negative Masson's stain, indicating the absence of hepatic fibrosis (Fig. 5K). However, Examination of the liver tissue of rats administered with MSCs in the portal vein showed a large focal area of markedly positive blue Masson's stain, indicating severe hepatic fibrosis (Fig. 5L).



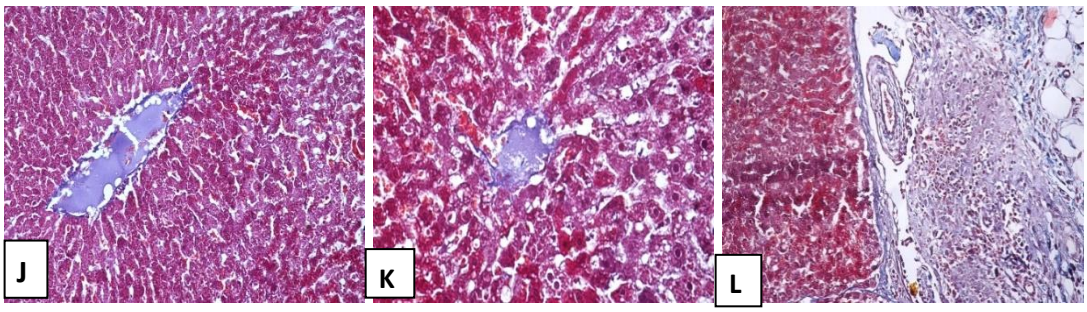


Fig. 5A: Liver from **the control group** showing negative Masson's stain indicating absence of hepatic fibrosis (Masson's X400). **Fig. 5B:** Liver from **group II treated with GalN** showing multi-focal areas of marked positive bluish Masson's stain indicating severe hepatic fibrosis (f) (Masson's X200). **Fig. 5C:** Another view of liver from **group II treated with GalN** showing diffusely marked positive bluish Masson's stain indicating severe hepatic fibrosis (f), (Masson's X200). **Fig. D:** Liver from **group III** treated with **MSCs after 2 hours** showing positive bluish Masson's stain in the portal area indicating portal area fibrosis (arrow), hepatic parenchyma showing no fibrosis (Masson's X200). **Fig. E:** Liver from **group IV** treated with **MSCs after 24 hours** showing positive bluish Masson's stain in the portal area, portal indicating portal area fibrosis (arrow), hepatic parenchyma showing no fibrosis (Masson's X400). **Fig. F:** Liver from **group V** treated with **MSCs after 48 hours** showing negative Masson's stain indicating absence of hepatic fibrosis (Masson's X400). **Fig. G** Liver from **group VI** treated with **MSCs after 72 hours** showing positive bluish Masson's stain in the portal area, indicating portal area fibrosis (arrow), hepatic parenchyma showing no fibrosis (Masson's X200).. **Fig. H:** Liver from **group VII** treated with **MSC Fraction for 4 days** showing negative Masson's stain indicating absence of hepatic fibrosis (Masson's X400). **Fig. I:** Liver from **group VIII** treated with **EPO (12 IU/kg IP)** showing negative Masson's stain indicating absence of hepatic fibrosis (Masson's X400). **Fig. J:** Liver from **group IX** treated with **EPO (12 IU/kg IP) + MSCs,** showing negative Masson's stain indicating absence of hepatic fibrosis (Masson's X400). **Fig. K:** Liver from **Group X** treated with **EPO (12 IU/kg IP) + 4 MSC fractions,** showing negative Masson's stain indicating absence of hepatic fibrosis (Masson's X400). **Fig. 5L:** Liver from **Group XI** treated with **MSCs administration in portal vein** showing large focal area of marked positive bluish Masson's stain indicating severe hepatic fibrosis (f) (Masson's X200).

DISCUSS ION

Various agents can induce acute liver injury, with GalN being one of the notable ones, partly due to its interactions with the immune system. GalN's hepatotoxicity primarily arises from the depletion of UDP-sugars, particularly UDP-galactose and UDP-glucose, coupled with the release of pro-inflammatory mediators like TNF-α from Kupffer cells. This toxicity

is further exacerbated by the disruption of intracellular calcium balance and the alteration of the uridine pool within hepatocytes **(Izu *et al*., 2007)**.

These modifications affect the synthesis of both proteins and nucleic acids, as well as cellular membranes and organelles. GalN alters the phospholipid composition of cellular membranes, compromises the enzymes responsible for substrate transport to mitochondria, and disrupts hepatocyte energy metabolism at elevated concentrations (**Tawfik *et al*., 2015)**.

The BCL-2 gene family is recognized for encompassing genes that encode both pro-apoptotic proteins, such as BAX and BAK, as well as anti-apoptotic proteins, including BCL-2 and BCLX ***(Kotipatruni et al., 2011)***.

BCL-2, a 26-kDa protein localized within mitochondrial membranes, is known for its role in inhibiting apoptosis when overexpressed (Liu et al., 2011-a). EPO administration has demonstrated anti-apoptotic effects in both in vivo and in vitro studies. In EPO-treated animals, a reduction in GalN-induced BAX upregulation was observed. In vitro, EPO's anti-apoptotic function has been well established, showing downregulation of apoptotic genes like BAX and caspase-3, alongside upregulation of anti-apoptotic genes such as BCL-2 and STAT5. The resulting increase in the BCL-2/BAX ratio is a reliable marker of apoptosis inhibition. Furthermore, the constitutive activation of STAT5 leads to enhanced cell proliferation and diminished apoptosis, playing a significant role in cancer initiation and progression (Valentine and Pierre, 2006). EPO has been shown to enhance the phosphorylation of STAT5 and modulate its nuclear translocation, leading to an increased expression of BCL-XL and a reduction in caspase-3 levels (Ma et al., 2014). These findings align with our in vitro results, which demonstrate a significant increase in cell proliferation assays in EPO-treated cultures compared to those without EPO treatment. The proliferative effects of EPO may be attributed to the upregulation of STAT5, an increase in the anti-apoptotic BCL-2/BAX ratio, and its anti-inflammatory properties.

Furthermore, EPO exerts tissue-protective effects by inhibiting NF-κB activation and decreasing the production of proinflammatory mediators, such as TNF-α, IL-6, IL-12/IL23 subunits, and NO, through inducible NO synthase (iNOS) in macrophages (Nairz et al., 2012).

The persistent activation of STAT-5 leads to enhanced cellular proliferation and diminished apoptosis, positioning it as a critical factor in the initiation and progression of cancer ***(Valentino and Pierre, 2006)***. EPO has been shown to enhance STAT5 phosphorylation and further modulate the nuclear translocation of phospho-STAT5, resulting in upregulated expression of BCL-XL and a concomitant reduction in caspase-3 levels ***(Ma et al., 2014)***. These findings align with our in vitro results, which demonstrate a significant enhancement in the proliferation assays of EPO-treated cell cultures compared to those not treated with EPO. The proliferative effects of EPO may be attributed to the upregulation of STAT5, an increase in the BCL-2/BAX anti-apoptotic ratio, and its anti-inflammatory properties. EPO confers tissue protection by inhibiting NF-κB activation. Additionally, EPO disrupts the production of pro-inflammatory mediators, including TNF-α, IL-6, IL-12/IL-23 subunits, and nitric oxide (NO) through the suppression of iNOS in macrophages ***(Nairz et al., 2012)***.

In the current study, injection of GalN showed very high levels of IL-6 when compared to the control group. However, MSCs and EPO- treated group showed a marked improvement in comparison with the group treated with MSCs or EPO alone. This observation comes in agreement with the results of (**Xagorari *et al*., 2013).** IL-6 is a particularly intriguing cytokine due to its dual role in both pro- and anti-inflammatory processes. The IL-6 signaling pathway facilitates inflammatory responses by activating and proliferating lymphocytes, promoting B cell differentiation, and recruiting leukocytes to the liver (Stenvinkel et al., 2005). Notably, serum IL-6 levels are closely associated with sepsis severity. Moreover, IL-6 may potentiate the TNF-α-induced apoptosis in hepatocytes that have been sensitized by galactosamine exposure (**Aristatile *et al*., 2013).**

Oxidative stress, beyond causing direct cellular damage, can also activate transcription factors such as NF-κB, which governs the expression of various inflammatory genes involved in hepatotoxicity (Lingappan, 2018). NF-κB, along with its target genes, plays a crucial role in modulating immune and stress responses, influencing key cellular processes including apoptosis, proliferation, differentiation, and development (Oeckinghaus and Ghosh, 2009). The activation of NF-κB triggers the release of inflammatory cytokines, including IL-1, IL-6, IL-12, IL-1β, and TNF-α (Zhan et al., 2014).

The findings of this study demonstrated highly increase in NF-κB gene expression in GalN-injected group compared to control. However, synergetic effect of both MSCs and EPO appeared by marked decrease in NF-κB gene expression. This in agreement with the results of (**Kim *et al.*, 2015).**

GalN causes hepatic damage via up regulation apoptosis- involved genes including BAX gene **(Raj *et al*., 2010)**. This correlates with our results that show up regulation of BAX gene in GalN- injected group compared to control group, suggesting that, apoptosis may be involved in GalN-induced hepatic toxicity through activation of BAX gene. Our results reveal up regulation of iNOS gene expression in response to GalN toxicity compared to the control group. This agrees with the results of **(Bultinck *et al*., 2006).** However, MSCs-treated group down-regulate iNOS mRNA raised by GalN toxicity with explanation of anti-inflammatory effect of MSCs. This outcome agrees with the results of **(Cho *et al*., 2014)**. In addition, EPO alone or with MSCs decreased iNOS expression in GalN injured rats. These findings are consistent with those reported by Sharawy et al. (2015). It is important to note that NO is a well-established pro-inflammatory mediator implicated in the pathogenesis of inflammation (Yang et al., 2010). While NO produced by iNOS has demonstrated beneficial effects, including microbiocidal, antiviral, antiparasitic, and antitumoral activities, prolonged NO production can be harmful to the host and has been associated with the development of various inflammatory diseases (Kanwar et al., 2009). The cytokine-induced expression of iNOS is significantly involved in the pathogenesis of inflammatory disorders, such as toxin-induced liver injury. Research on iNOS-deficient mice and studies employing iNOS inhibition have highlighted the role of inducible NO in regulating a wide array of processes, including leukocyte recruitment and adhesion, inflammatory diseases, wound healing, ischemia, and tumor-induced angiogenesis (Cassini-Vieira et al., 2015).

GalN causes hepatic damage via up regulation apoptosis- involved genes including Bax gene **(Raj et al., 2010)**. This correlates with our results that show up regulation of Bax gene in GalN- injected group compared to control group. Suggesting that, apoptosis may be involved in GalN induced hepatic toxicity through activation of BAX gene.

The findings of this study indicates upregulation in the gene expression of apoptosis proteins such as Bax in the GalN group when compared to the control group. This come in agreement with the results of the (Orazizadeh, *et al*.2020). Suggesting that, apoptosis may be involved in GalN induced hepatic toxicity through activation of Bax gene. While, MSCs treated group inhibit apoptosis by controlling the effects of Bax / Bcl-2 this come in agreement with the results of the (Zare, *et al*. 2019).

MSCs are recognized for their potent immunomodulatory, anti-inflammatory, and anti-apoptotic characteristics, which operate through paracrine or endocrine mechanisms to facilitate tissue repair. These mechanisms function by attenuating the inflammatory response and reducing the expression of apoptotic markers such as caspase-3 and Bax [Zare, *et al*. 2019]

Examination of the sections of the liver post GaIN injection (**group II)** showed multi-focal areas of marked positive bluish Masson's stain indicating severe hepatic fibrosis. While, EPO plus MSCs administration also treatment with EPO plus MSC fractions showing negative Masson's stain indicating absence of hepatic fibrosis. On contrast to the MSCs administration in portal **vein** showing large focal area of marked positive bluish Masson's stain indicating severe hepatic fibrosis. According to this study, injecting MSCs into the tail vein is more effective than doing so into the portal vein.

Conclusion

The current study indicates that EPO promotes the recruitment of BMSCs to areas of liver cell injury, upregulates the expression of Bcl-2, STAT-5, and NFκB, thereby enhancing the anti-apoptotic effects of BMSCs, and expedites the restoration of liver function after acute liver failure.

Conflicts of interest

Non.

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