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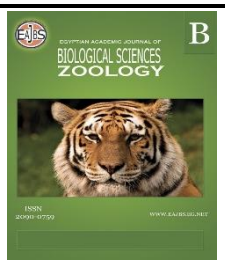

Mesenchymal Stem Cells and Erythropoietin Therapy in Acute Liver Failure Induced by D-galactosamine in Rats

Reda Hassan[1], Samir A. M. Zaahkouk[1], Mansour A.[2], Mahmoud Ashry[3] and Sawsan El-Shamy[4]

[1]Zoology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt.
[2]Pharmacology Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.
[3]Zoology Department, Faculty of Science, Al-Azhar University, Assiut, Egypt.
[4]Biology Department, Basic Science Center, Misr University for Science and Technology, Cairo, Egypt.

*** E-mail** : dr8648368@gmail.com ; elshamysawsan41@gmail.com

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ABSTRACT

Erythropoietin (EPO), in addition to its hematopoietic functions, demonstrates non-hematopoietic functions such as anti-apoptotic, anti-inflammatory, and antioxidant properties. The EPO effect was confirmed to be tissue-protective in the context of myocardium, brain, kidney, and liver injuries. Mesenchymal stem cells (MSCs) are non-hematopoietic cells. It can be extracted from the dental pulp, bone marrow, placenta, adipose tissue, amniotic membrane, or umbilical cord. MSCs have the potential to repair damaged tissues of the liver, enhance liver functions, and decrease fibrosis of the liver, as evidenced by preclinical and clinical research studies. **Materials and Methods:** Eleven equal groups were created from sixty-six mature male albino rats. There were some biochemical investigations conducted on blood samples, and histological studies were assessed in the liver tissue. **Results:** The toxic effect of GalN was mitigated by MSCs and EPO treatment, resulting in a substantial reduction in the mean levels of AST, ALT, and total bilirubin and alkaline phosphate, an improvement in albumin level, and histological studies. The protection against GalN toxicity was more efficacious when MSCs and EPO were combined.

INTRODUCTION

Acute liver failure (ALF) is a highly fatal clinical illness that is typified by a sharp decline in liver function in a brief amount of time, even in the absence of underlying chronic liver diseases (EASL, 2017). The best treatment option for people with ALF is liver transplantation, which improves their prognosis. However, the scarcity of donor organs and the high expense of medical care prevent liver transplantation from being widely used. (Dogan and Gurakar, 2015). Therefore, there is a critical need for innovative therapeutic techniques for ALF. In recent times, Stem cell transplantation has recently provided a different approach for patients to prolong their lives (Zhang *et al.*, 2022).

Hepatotoxins like galactosamine (GalN) are well known. In animal models of liver injury, a single injection of GalN and repeated injections can cause acute hepatitis and

chronic liver failure, respectively (Wang *et al.*, 2013).

Stem cell transplantation has recently provided a different approach for patients to prolong their lives (Zhang *et al.*, 2022). including chondrocytes, adipose cells, osteocytes, and hepatocyte-like cells. MSCs take part in immunomodulation and release soluble cytokines (Hu *et al.*, 2020). They can be extracted from a number of different human body locations, including the dermis, adipose tissue, bone marrow, and umbilical cord (UC) (Fan *et al.*, 2020). Given its advantages of having an abundance of resources, being simple to collect, and having a noninvasive collection process, the UC is a popular source for MSC isolation. (Nagamura-Inoue and Nagamura, 2023)

Research conducted in both preclinical and clinical settings has demonstrated that mesenchymal stem cells (MSCs) originated from various origins have the ability to repair damaged liver tissues, enhance liver function, and decrease liver fibrosis Kim *et al.* (2019)

For application to be successful, the target tissues need to home a significant quantity of MSCs following administration. However, the inability of MSCs to be successfully homing after systemic injection is a major disadvantage of MSC treatment (Yuan *et al.*, 2022).

The glycoprotein hormone erythropoietin (EPO), which is mostly produced in the kidneys, promotes the growth and maturation of progenitor cells responsible for the production of red blood cells. The kidneys are safe guarded by EPO. However, The information gathered suggests that EPO serves a defensive role in the neurological system (Auzmendi *et al.*, 2020), anti-oxidation (Chen *et al.*, 2015), anti-inflammation (Nakamura *et al.*, 2015), and apoptosis (Li *et al.*, 2020), since the erythropoietin receptor (EPOR) is found in multiple systems in addition to the kidneys. EPO pretreatment enhances MSC quality due to EPOR dispersion on MSCs, which is advantageous when employing MSCs to treat ulcers (Lu *et al.*,2016). Nevertheless, it can be challenging to obtain optimal MSC quality following a single therapy because of the brief half-life of EPO. In this work, we sought to examine the possible impacts of MSCs and EPO on the hepatic toxicity caused by galactosamine, as well as the potential mechanisms of action of MSCs treated with EPO for varying lengths of time and different routes of administration in the treatment of liver disease.

MATERIALS AND METHODS

1-Experimental Animals:

Sixty-six male Sprague-Dawley rats weighing between 200 and 220 g, were maintained in the animal house as established by Institutional Animal Ethical Committee (IAEC), in accordance with the established protocols of the Al-Azhar University, Faculty of Science's ethical committee. The animals were housed under good conditions of temperature, light, good ventilation, standard normal diet and water.

Experimental Design:

The animals were allocated into 11 groups, each of which contained 6 rats:

Group I: It served as a healthy control group and received an injection of saline.

Group II: In order to elicit acute hepatic toxicity, rats were injected intraperitoneally (IP) with GalN (1700 mg/kg IP) in a single dose dissolved in saline. (Bigoniya *et al.*, 2009).

Group III: Two hours following the GalN injection (1700 mg/kg IP) dissolved in saline, the rats were injected with MSCs at a concentration of one million cells/rat in the caudal vein (Volarevic *et al.*, 2014).

Group IV: Rats were injected with one million MSC cells/rat after 24 hours, following GalN injection. (1700 mg/kg IP) dissolved in saline Lipsic *et al.* (2008).

Group V: One injection of one million MSC cells/rat to rats immediately after the GalN injection (1700 mg/kg, IP) within 48 hours.

Group VI: MSCs (one million cells/rat) were administered to rats once within 72 hours of the GalN administration (1700 gm/kg) IP.

Group VII: Immediately following the GalN injection, (1700 mg/kgIP) rats were administered MSCs of one million cells/rat fractionated (250 cells/rat) for four days.

Group VIII: After receiving an injection of GalN, rats received a single injection of EPO (12 IU/kg IP). (1700 mg/kg IP) as modified from Lipsic et al. (2008).

Group IX: Immediately after receiving GalN injection (1700 mg/kg IP), rats were given MSCs (one million cells/rat) and EPO (12 IU/kg IP).

Group X: Immediately following the GalN injection (1700 mg/kg IP), rats were administered EPO (12 IU/kg IP) and one million MSCs fractionated (250 cells/rat) for a period of 4 days.

Group XI: One million MSCs were infused into the rats' portal veins right after they received a GalN injection (1700 mg/kg IP). Via the tail vein, males received an intravenous injection of 25 mg/kg thiopental to induce anesthesia. Making a midline incision, the left side of the abdominal wall's skin was carefully separated from the underlying muscle while being careful not to bleed. The linea alba was incised to reveal the abdomen. With the division of the delicate peritoneal interconnections between the spleen and the stomach, the spleen retained full mobility on its pedicle. Using a 26-cannula that was attached to the syringe, fresh stem cells and/or erythropoitein were fed into the portal vein over the course of five minutes. After that, the cannula was removed from the portal vein. A subcuticular catgut suture was used to close the skin of the abdomen, while a continuous catgut stitch was used to close the muscle layer.

Blood and Tissue Sampling:

After thirty days of all the treated groups. Venous blood was collected from the retro-orbital vein from rats of all groups, and animals were sacrificed by cervical dislocations.

After overnight fasting, then post-anesthesia, venous blood was drawen from the retro-orbital vein from all groups of rats (after thirty days of treatment) , allowed to clot, and then cool-centrifuged. In order to facilitate as quickly as possible biochemical tests, the sera were stored at -80°C in aliquots after being separated. Following the collection of blood, As soon as the animals were sacrificed, the animals' livers were taken and immersed in a 10% formalin-saline buffer for processing histopathologically and histochemically.

Biochemical Determinations:

Serum ALT and AST activities were measured using the techniques of Young (2000) and Young (1997), respectively, and alkaline phosphatase (ALP) activity according to the method of Friedman and Young (1997). Total bilirubin was estimated (Tietz, 1995), and albumin levels were estimated spectrophotometrically using the method of Tietz (1990). All purchased from Biodiagnostic Co., Dokki, Giza, Egypt.

Histopathological Study:

The histology studies were done in accordance with Bancroft and Steven (2013). Thyroid tissues were, in short, cut to a thickness of 3–4 mm, dehydrated in varying ethanol concentrations, cleared in xylene, and stained with masson trichrome stain before microscopic examination.

2-*In Vivo* Study:

a) Isolation, Propagation, Labeling, And Identification of Bone Marrow-Derived Mscs from Rats: Rats were scarified by cervical dislocation, and the bone marrow was removed. Rats' femurs and tibias were removed under sterile conditions, and extra care was taken to remove any connective tissue that was linked to the bones. By flushing the tibiae and femurs with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) enhanced with 10% fetal bovine medium (GIBCO/BRL), bone marrow was extracted. Using a density gradient [Ficoll/Paque (Pharmacia)], nucleated cells were separated and then resuspended in full culture media that was enhanced with 1% penicillin-streptomycin (GIBCO/BRL). For 12–14 days, cells were cultured at 37°C in an incubator with 5%

humidified CO2 to create big colonies. Following two rounds of phosphate buffered saline (PBS) washing, cultures were trypsinized with 0.25% trypsin in 1 mM EDTA (GIBCO/BRL) for five minutes at 37 °C. This was done once substantial colonies (80–90% confluence) had formed. Following centrifugation (for 20 minutes at 2400 rpm), cells were resuspended in media supplemented with serum and incubated in a Falcon 50 cm 2 culture flask. First-passage cultures are what were left behind (Alhadlaq and Mao, 2004). On day 14, the adherent cell colonies were trypsinized and counted. Figure 1(a, b)

b) Labeling of Stem Cells with PKH26 Dye: PKH26 fluorescent linker dye was used to identify MSC cells that were extracted during the fourth passage (Figure 1(C)). Red fluorochrome PKH26 has emission at 567 nm and excitation at 551 nm. The linkers have negligible to no harmful side effects on cell systems and are physiologically stable. For in vitro cell labeling, in vitro proliferation experiments, and long-term in vivo cell tracking, labeled cells are perfect since they maintain both biological and proliferative activity.

Labeled cells that have been rinsed can be seen in culture (for non-dividing cells). Up to 100 days after labeling The improved stability is advantageous for long-term in vivo research. Depending on the initial staining intensity and the surface area of the cells, up to eight divisions can be seen after staining with PKH dyes. The most common divisions that can be seen are 4-6. As directed by the manufacturer (Sigma pkh26gl), final concentrations of 2 × 10-6 M PKH26 dye and 1 x 107 cells/ml in a 2 ml total volume were stained. Undifferentiated MSCs were administered intravenously into the rat tail vein following PKH26 labeling.

c) Morphological Identification of BM-derived MSCs: The cultures' morphology was examined using transmission electron and optical microscopy. MSCs in culture were distinguished by their fusiform shape and adhesiveness.

d) Flow Cytometry Identification of Cells: After being cleaned, MSCs were again suspended in phosphate-buffered saline. Monoclonal antibodies against CD29 and CD34 (Santa Cruz Biotechnology, Santa Cruz, CA) were applied straight to the cells and incubated for one hour at 4 °C. Following that, the cells were treated for 45 minutes on ice with anti-mouse immunoglobulin G fluorescein conjugated with a secondary antibody (Millipore Corp., Temecula, CA). After two washings, cell suspensions were examined using a flow cytometer with a FACS caliber.

e) Detection of the Homing of Injected Cells in Rat Liver Tissue:

A fluorescent microscope was used to examine liver tissue after a week in order to follow the injected cells throughout the tissue and find the cells dyed with PKH26 dye, which ensures homing.

Statistical Analysis:

The presentation of all data is as the mean ± standard error of the mean. Tukey's multiple comparison tests and one-way analysis of variance were used to assess the results. Statistics were deemed to show a statistically significant difference at a value of $P<0.05$.

RESULTS

***In vitro*:**

1-MSCs Isolation, Propagation, Identification and Labelling:

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 1b**).** For better MSCs identification inside liver tissue, *The* effective homing of MSCs into liver tissue was confirmed when they were observed in the tissue after being labeled with the fluorescent dye PKH26.

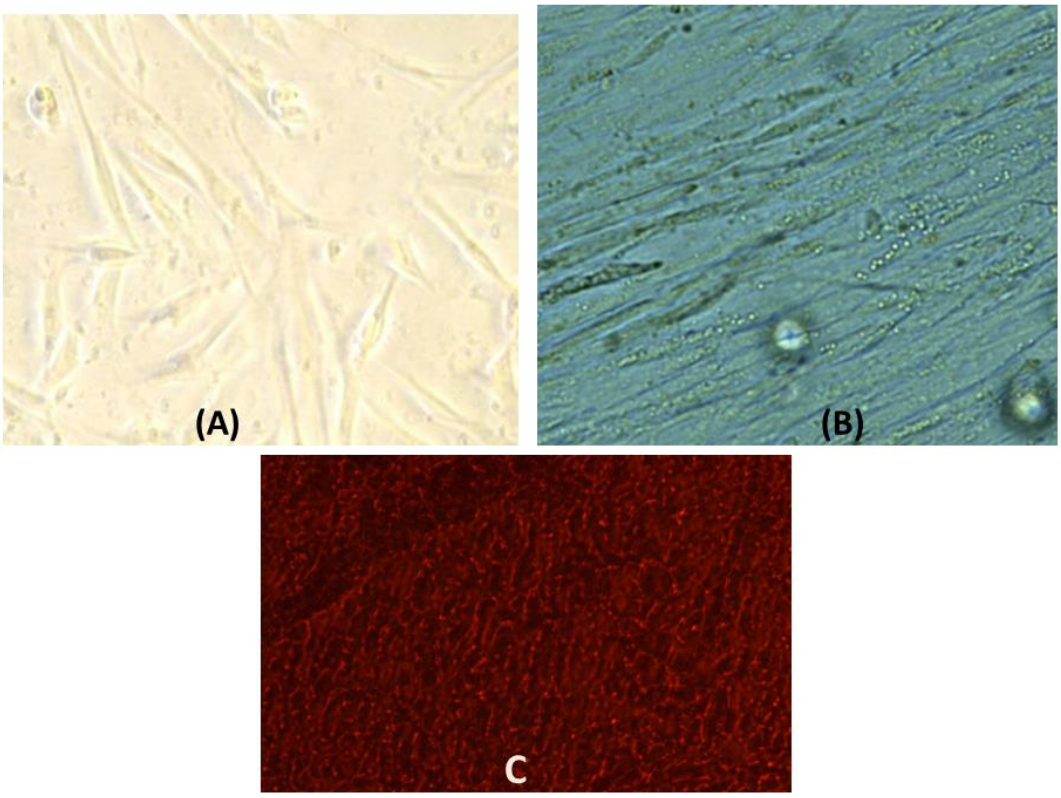


Fig. 1 (A): Spindle-shaped MSCs at one-week culture. (B) MSC morphology at 2 weeks of culture. ***(C)*** MSCs are labeled using the PKH26 dye.

The presence of MSCs stained with PKH26 fluorescent dye in the liver tissue provided evidence that the cells had migrated into the liver tissue.

Analysis of MSCs Based on Cell Surface Marker Expression:

Using flow cytometry, the surface markers of MSCs (CD34 and CD29) were examined, and the results revealed that the MSCs were uniformly negative for CD34 (Fig. 2 a) and positive for CD29 (Fig. 2 b).

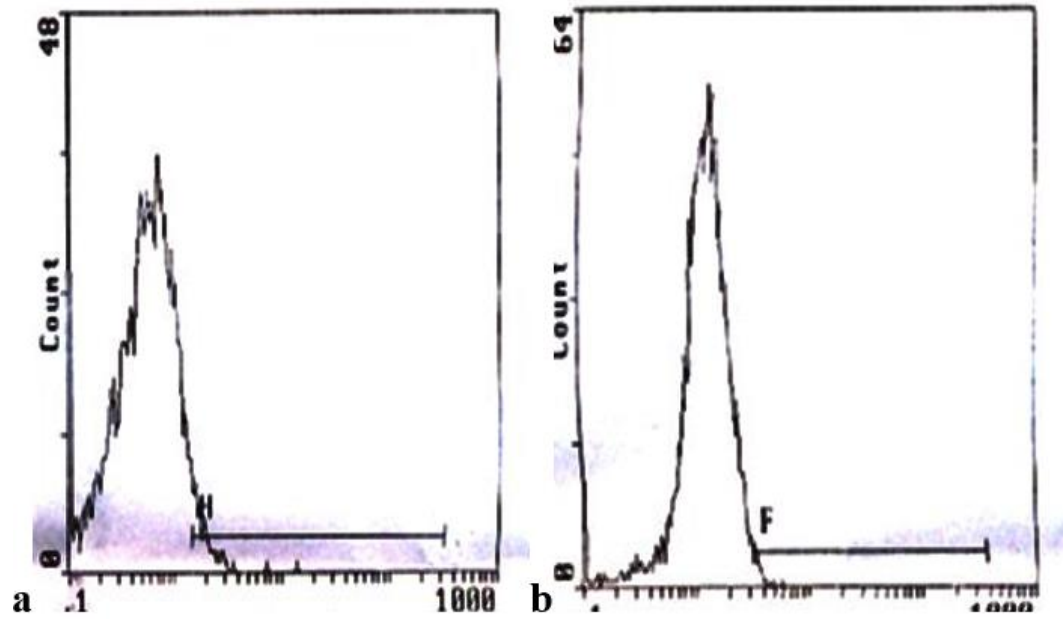


Fig. 2: Flow cytometric characterization analyses of bone marrow-derived MSCs. (a) Cells were uniformly negative for CD34 (b) and positive for CD29.

Biochemical Results:

The results of the present investigation demonstrated that there was a noticeable increase in the ALT, AST, and ALP activities. (*; *P <0.01)* in the GalN group versus control group figure 3 (A, C, and E), indicating severe liver injury. However, we found that treatment with MSCs and/or EPO for various periods reduced the increases in ALT, AST, and ALP to varying degrees when compared to the GalN group. It was found that the **G-X** group that was treated with EPO + MSC fractions for 4 days gave the best improvement result. Also, figure 3(B, D, and F) showed the impact of the time of treatment with MSCs on the enzyme activities. It was found that the fractionated treatment with MSCs for 4 days (250 cells/day) results in highly *improved activity* of these enzymes. Indicating the highly ameliorating effect of MSCs when combined with EPO. Also, there are various effects of the time of treatment with MSCs alone on the activities of the liver enzymes.

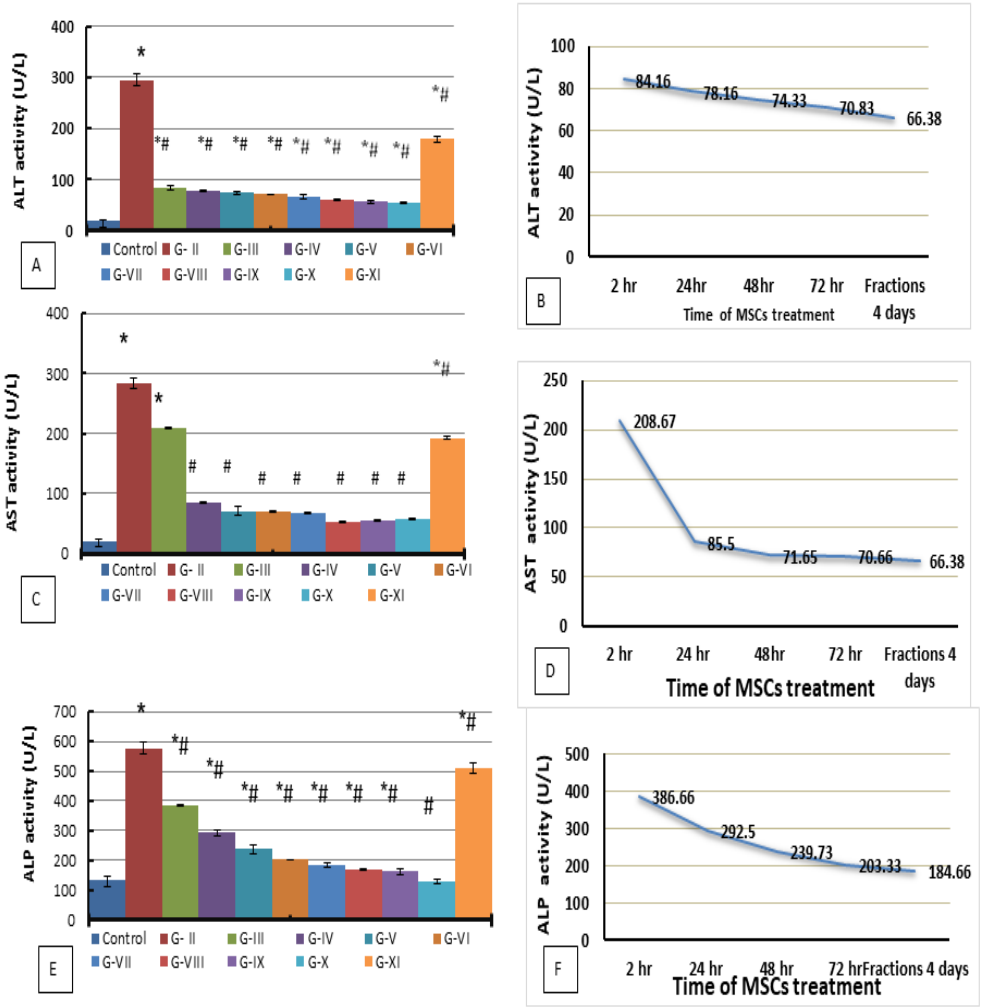


Fig. 3 (A, B, C, D, E, and F): Impact of MSCs and/or EPO against GalN-induced alterations at activities of serum ALT, AST, and ALP in the control and different treated groups. *: Significant ($p < 0.001$) in comparison to the control group. #: Significant ($p < 0.001$) relative to GalN-treated groups. Data were represented as mean±SE (n = 6) figures (A. C. and E). **G-II (**GalN), **G-III** (MSCs after 2 hr), **G-IV** (MSCs after 24 hr), (MSCs after 48 hr), **G-V** (MSCs after 72 hr), **G-VII** (MSCs Fraction for 4 days), **G-VIII** (EPO), **G-IX** (EPO + MSCs), **G-X** (EPO + MSC fractions for 4 days). **G-XI** (MSCs portal vain). Effect of time of MSCs administration (after two, four, eight, and twelve hours, and fractions for 4 days) on each enzyme activity figure (B, D, and F).

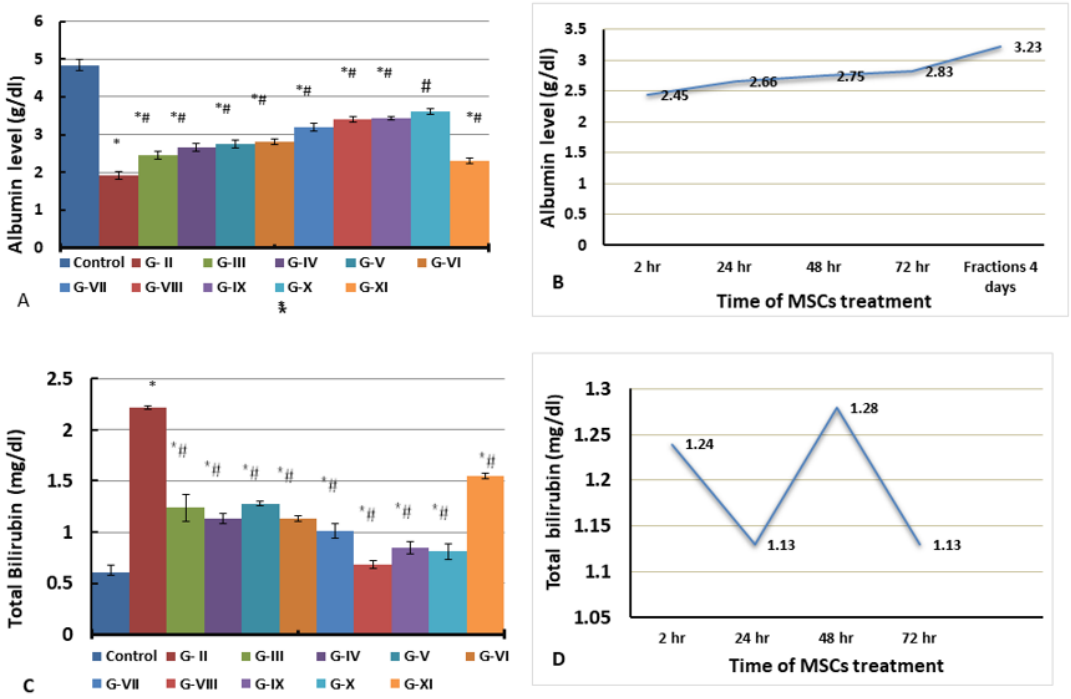
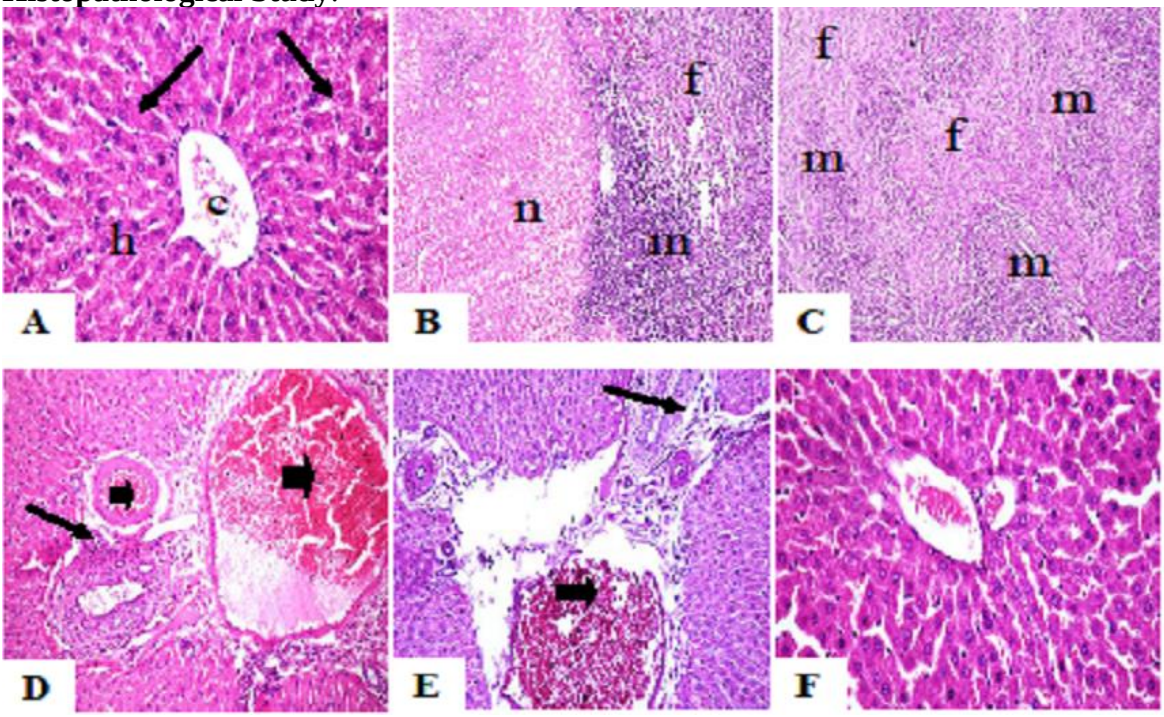


Fig. 4 (A, B, C, and D): Effect of MSCs and/or EPO against GalN-induced alterations at levels of serum albumin and total bilirubin in the control and the different studied groups. *: Significant ($p < 0.001$) in comparison to the control group. #: Significant ($p < 0.001$) relative to GalN treated groups. Data were represented as mean±SE (n = 6) (figures **A and C**). **G-II (**GalN), **G-III (MSCs** after 2 hr), **G-IV** (MSCs after 24 hr), (MSCs after 48 hr), **G-V** (MSCs after 72 hr), **G-VII** (MSCs Fraction for 4 days), **G-VIII** (EPO), **G-IX** (EPO + MSCs), **G-X** (EPO + MSC fractions for 4 days). **G-XI** (MSCs portal vain). Effect of time of MSCs administration (after 2 hr, 24 hr, 48 hr, 72 hr, and fractions for 4 days) on serum albumin and bilirubin levels (Figs. **B and D**).

Histopathological Study:



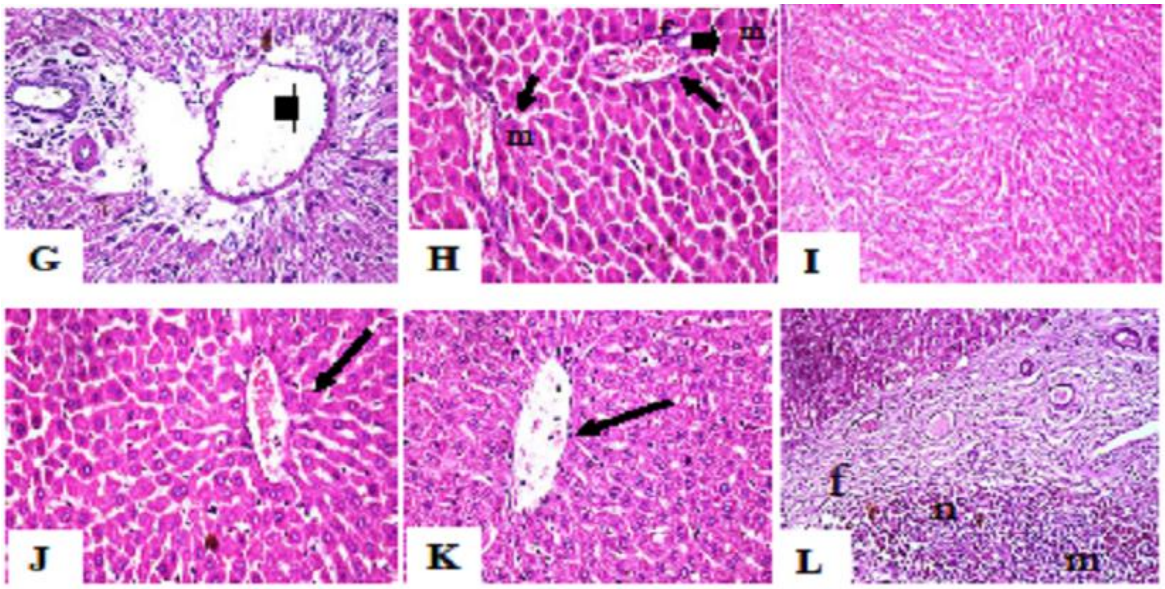


Fig. 5: Showed that H&E was used to stain the liver tissue in order to evaluate the impact of MSCs and/or EPO on liver damage induced by GalN injection. The control group displayed a normal appearance of hepatic parenchyma; observe the hepatic cords that are arranged and include normal polygonal hepatocytes (h) **(Fig. 5A).** While the histological examination of the liver tissues showed that GalN injection caused severe hepatic necrosis (n), fibrosis (f), together with mononuclear cell infiltration (m) **(Figs. 5B and C).** Liver from group III treated with MSCs after 2 hours showing markedly improved apparently healthy hepatic parenchyma and dilatation & congestion of the hepatoportal blood vessels (arrow head), together with hyperplasia in the bile duct (arrow), hepatic parenchyma showing no fibrosis (**Fig. 5D**). Liver from Group IV trated with MSCs after 24 hours showing markedly improved apparently healthy hepatic parenchyma and dilatation & congestion of the hepatoportal blood vessels (arrow head), together with hyperplasia in the bile duct (arrow), hepatic parenchyma showing no fibrosis (**Fig. 5E**). Liver from Group V treated with MSCs after 48 hours showed markedly improved apparently healthy hepatic parenchyma, hepatic parenchyma showing no fibrosis (**Fig. 5F**). Liver from Group VI treated with MSCs after 72 hours showing moderately improved hepatic parenchyma, together with dilatation without congestion of the hepatoportal blood vessels (arrowhead), hepatic parenchyma showing no fibrosis (**Fig. 5G**). Liver from Group VII treated with MSC fractions for 4 days showed markedly improved apparently healthy hepatic parenchyma and normal portal areas (arrows), hepatic parenchyma showing no fibrosis (**Fig. 5 H**). Liver from Group VIII treated with EPO (12 IU/kg IP) showing moderately improved hepatic parenchyma, hepatic parenchyma showing no fibrosis (**Fig. 5 I**). Liver from group IX treated with EPO (12 IU/kg IP) + MSCs showing markedly improved apparently healthy hepatic parenchyma, together with slight congestion of central vein (arrow), hepatic parenchyma showing no fibrosis (**Fig. 5J).** Liver from group X trated with (EPO (12 IU/kg IP) + MSC fraction) showing markedly improved apparently healthy hepatic parenchyma and normal central vein (arrow), hepatic parenchyma showing no fibrosis (**Fig. 5K**). Liver from Group XI treated with MSCs in the portal vein shows a large focal area of hepatic necrosis (n) and fibrosis (f), together with mononuclear cell infiltration (m) (**Fig. 5L).**

DISCUSSION

Numerous agents induce acute liver injuries. GalN is one of them. The immune system appears to be partially included in the process via which GalN causes hepatic damage, despite the fact that it is not well understood. Hepatocytes' ability to synthesize

macromolecules is inhibited by GalN, a particular hepatotoxin that causes selective depletion of uridine nucleotides. This leads to anomalies in the composition and activity of hepatic cells (Kuhla *et al.,* 2009).

Lipid peroxidation results from the respiration and degranulation of activated neutrophils, which gather around injured liver cells and release oxygen-free radicals. Liver parenchyma and vascular endothelial cells are the targets of oxygen free radicals and lipid peroxidation, which causes cell damage or death (Jaeschke, 2011).

These modifications impact the structure of cell membranes, the functioning of organelles, and the production of nucleic acids and proteins. At larger doses, GalN suppresses the metabolism of hepatocytes, disrupts the enzymes responsible for transporting substances to the mitochondria, and alters the composition of phospholipids in cell membranes (Tawfik *et al.,* 2015).

MSC therapy in conjunction with other therapies may actually work better for ALF than MSC therapy alone (Sang *et al.,* 2023).

With their capacity for self-replication, high proliferation, and multidifferentiation, stem cells are a type of cell that can have a variety of therapeutic effects, including tissue healing and immunomodulation (Shang *et al.,* 2021). Since mesenchymal stem cells (MSCs) have an abundant supply, minimal immunogenicity, and no ethical constraints, they are frequently employed in research on disease treatment (Ankrum *et al.,* 2014). According to earlier research, MSCs regulate immune cells, secrete healing factors, and differentiate into hepatocyte-like cells (HLCs) to treat ALF **(**Hu *et al.,* 2020).

The promise for utilization of MSCs in regenerative medicine is demonstrated by their ability to regenerate bone, adipocytes, endothelium cells, muscle cells, and neurons (Wei *et al.,* 2013). Because MSCs express few classes I major histocompatibility molecules and lack major histocompatibility antigens of class II, they are hypoimmunogenic (Caplan., 2009).

MSC-derived hepatocyte-like cells offer a promising supply of liver-regenerating cells. When MSCs are cultured with cytokines, such as IL-6, growth factors, leukocyte inhibitor, fibroblast growth factor, hepatocyte growth factor, epidermal growth factor and so on, they can develop into hepatocyte-like cells (Afshari *et al.,* 2020). Adipose-derived (AD)-MSCs were shown to differentiate into hepatocyte-like cells that displayed the functional traits of hepatocytes, such as the expression of albumin, the secretion of urea, the activity of cytochrome P450, the storage of glycogen and the uptake of low-density lipoprotein (Okura *et al.,* 2010).

Because of their engraftment ability, transdifferentiation potential, or cell fusion (MSCs can fuse with other cells to form syncytiums, which are multinuclear cells) (Liang *et al.,* 2014). Liver toxicity induced by GalN can be rectified by MSCs. Although numerous researches have indicated that recombinant human EPO has the powerful to improve liver function and promote liver regeneration as a result of its multifunctional cytokine properties, which include mitogenic, tissue-protective, and anti-apoptotic properties (Peng *et al.,* 2014).

It has been suggested that EPO is a pleiotropic hormone that protects tissue. By communicating via a nonhemopoietic receptor, EPO protects surrounding injury sites from tissue deterioration. (Brines and Cerami, 2005). Furthermore, it has been demonstrated that EPO binds to the heterodimeric erythropiotein receptor (EPOR) in order to exert its extrahematopoietic effects (Brines *et al.,* 2004).

In the present study, hepatocyte damage induced by D-GalN in rats leads to increased serum ALT, AST, and ALP activities and serum total bilirubin levels versus the control group.Treatment with MSCs and/or EPO groups showed that the degree of liver tissue damage was ameliorated as the serum activities of ALT, AST, and ALP were reduced and bilirubin level was decreased in comparison with the groups treated with MSCs or EPO alone. Also, the group treated with MSCs at a fractionated dose for 4 days showed highly

improved activity of these enzymes versus the groups treated with a single dose of MSCs for two, twenty-four, forty-eight, and seventy-two hours.

However, the group that administered MSCs in the portal vein showed no improvement in the activities of ALT, AST, and ALP, serum total bilirubin, and albumin level, demonstrating the obvious effect of the route of administration.

The decreased serum levels of albumin in the GalN-group versus the control group increased near to the normal value in the groups treated with MSCs and/or EPO compared to the GalN group. This is consistent with a clinical experiment by Peng et al. (2011), where patients' albumin and total bilirubin levels dramatically improved following a single autologous bone marrow transplant.

Histopathological analysis of this study also showed that GalN injection caused severe hepatic necrosis, and fibrosis, together with mononuclear cell infiltration. The liver from the group treated with MSC fraction and EPO showed marked improvement, with apparently healthy hepatic parenchyma and a normal central vein; hepatic parenchyma showed no fibrosis than other treatment groups. This agrees with the result of YANG et al. (2014), who found that EPO treatment causes a reduction in necrosis in hepatocytes, haemorrhage, and inflammatory cell infiltration caused by GaIN. Also, Tao *et al.* (2024) found that the human umbilical cord mesenchymal stem cell treatment reduced pathological damage that resulted from GaIN toxicity.

This study showed injecting MSCs into the tail vein works better than the portal vein injection. As, administration of MSCs in the portal vein shows a big hepatic necrosis focal area and fibrosis, together with mononuclear cell infiltration, indicating the obvious effect of the injection route. These findings concur with those of Sun *et al.* (2014), who indicated the tail injection route of administration is effective in ALF treatment by MSCs. However, some studies come in contrast with this result that have demonstrated that using a portal vein for transplantation is more successful than using other methods (Cao *et al.*, 2012).

Although MSCs are effective in treating ALF, the application of MSCs in ALF needs to be further studied and optimized. We need further studies about the potential mechanisms of MSCs therapy and EPO for ALF at various periods of time and various routes of injection.

CONCLUSION

EPO-MSCs enhance anti-fibrotic efficacy, with higher cell viability and stronger migration ability compared with treatment with BM-MSCs only. The results of treatments vary according to the dose, period of treatment, and route of injection. These findings may contribute to the development of novel agents for the treatment of ALF. However, further investigations are required to determine the precise protective mechanism.

Declarations:

Ethical Approval: The animal experiments were approved by the ethical committe of the Faculty of Science, Al-Azhar University, Assiut, Egypt. (NO. 5/2024).

Competing interests: The authors have declared that no competing interests exist.

Authors Contributions. The submitted manuscript is the work of the author & co-author. All authors have contributed to authorship and have read and approved the manuscript. Approval of the version of the manuscript to be published: All authors revised and approved the manuscript content.

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