Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:739-760
DOI: 10.33594/000000473
Published online: 24 November 2021
Accepted: 1 November 2021

Original Paper

Evaluation of Mesenchymal Stem Cells with Conditioned Media and m-EGF for Regeneration of Liver Tissue After Partial Hepatectomy in Wistar Rats

Divya Mohan[a] Swapan Kumar Maiti[a] Shivaraju Shivaramu[a] Sangeetha Palakkara[a] Kalaiselvan Elangovan[a] Naveen Kumar[a] Bindhuja Belluti Venkateshappa[a] Surendra Dunthuru Siddaraju[a] Dinesh Murali[b] Sowbarenya Chelladurai[a] Ramith Kallahalli Rajanna[a] Jürgen Hescheler[c]

[a]Division of Surgery, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India, [b]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India, [c]Institute of Neurophysiology, University of Cologne, Cologne, Germany

Key Words
Partial hepatectomy • Liver regeneration • Stem cell • Histopathology • Immunohistochemistry

Abstract
Background/Aims: Liver is considered as the vital organ in the body as it performs various essential functions. Following an injury to the liver, the repair process even though initially beneficial becomes pathogenic when it is not controlled appropriately. Extensive accumulation of extracellular matrix (ECM) components can ultimately lead to cirrhosis and liver failure. Thus, the ideal strategy to treat a liver injury is to generate new hepatocytes replacing damaged cells without causing excessive ECM deposition. The objective of this study was to evaluate the potential of mesenchymal stem cells, conditioned media and murine epidermal growth factor (m-EGF) in liver regeneration following partial hepatectomy in a rat model. ***Methods:*** The animals were anaesthetized and a midline laparotomy was done. The liver was exposed and the left lateral and median lobes were ligated and resected out (about 65-70% of total liver mass). The muscles and skin were sutured in routine fashion and thus the rat model of partial hepatectomy was prepared. The animal models were equally distributed into 4 different groups namely A, B, C and D and treated with PBS, conditioned media, mesenchymal stem cells and epidermal growth factor respectively. The liver regeneration was assessed based on clinical, haemato-biochemical, colour imaging, histopathological and immune-histochemical parameters. ***Results:*** Partial hepatectomy model with surgical removal of 65-70% liver lobe was standardized and successfully used in this study**.** Alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), bilirubin, transaminases were significantly higher ($P<0.05$) in group A indicating that the liver damage was not restored properly**.** Colour digital imaging**,** histopathological and immune-histochemistry observations revealed that a better

Swapan Kumar Maiti,
Principal Scientist

Division of Surgery, ICAR-Indian Veterinary Research Institute,
Izatnagar, Bareilly, Uttar Pradesh 243122 (India)
Tel. +919412049169, E-Mail swapanivri@gmail.com

liver regeneration was observed in groups C and D, followed by groups B and A. Regeneration coefficient calculated based on liver weight was higher in groups C and D as compared to group A. ***Conclusion:*** Rat bone marrow-derived mesenchymal stem cells were found to induce hepatocytes proliferation; whereas EGF induced more angiogenesis. Conditioned media was not as effective as stem cells and EGF in liver tissue repair.

Introduction

The liver is an organ that plays a central role in maintaining metabolic homeostasis, in its functions of metabolism, synthesis and storage of nutrients. The cells in the liver are parenchymal cells and nonparenchymal cells. The former include hepatocytes (80% of the cell population) and the later, includes endothelial cells, Kupffer cells, lymphocytes and stellate cells. Liver dysfunction or failures are having different etiologies. Liver cancer has the fifth highest cancer incidence in the world, and is the third highest cause of cancer related deaths [1] with resection of the liver remaining the only curative option [2].

Hepatic resections are both associated with, and dependent on a rapid proliferation and regeneration of the remnant liver. But many patients experience temporary or permanent hepatic insufficiency after surgery, usually because they have factors potentially limiting liver regeneration, such as advanced age, steatosis, and liver cirrhosis [3]. Therefore, it is essential to optimize liver regenerative capacity in patients with suboptimal liver function or who are scheduled to undergo major hepatectomy. Exploring the newer scientific discoveries governing liver regeneration could contribute to enlightening our understanding of the mechanisms behind this process. This could offer new treatment strategies, not only to patients with advanced liver cancer, but also to patients suffering from acute and chronic liver failure, as the liver is the major detoxifying organ of the body and is likely to be injured by ingested toxins.

Orthotropic liver transplantation is a life-saving treatment for patients with end-stage liver disease, but it is limited by organ shortages, high expense, graft rejection, and the requirement for long-term immunosuppression and considerable long-term side effects that include chronic renal failure, post transplant lymphoproliferative disease, and cardiovascular complications [4].

The search for new therapies has been actively pursued for several decades, primarily in the form of artificial liver support devices and hepatocyte transplantation, yet both of these modalities remain experimental. During tissue injury, it has been suggested that bone marrow stem cells are mobilized and migrate to the injured organ to maintain physiologic hemostasis [5]. This theory has formed the basis for regenerative therapy whereby treatment with appropriate stem cells might ameliorate specific diseases. Eom et al. [6] has mentioned that mesenchymal stem cells (MSCs) can move toward areas of injury in response to signals of cellular damage, which are known as homing signals. This migration property of MSCs is important in regenerative medicine because various parenteral routes can be used depending on the damaged tissue or organ. Mesenchymal stem cells can be transplanted into the liver by intravenous, intraperitoneal, intrahepatic, intrasplenic, or portal-venous injection, however, the effects have differed based on their injection routes. MSC can differentiate into hepatocytes-like cells both *in vitro* and *in vivo* and can secrete trophic factors, including growth factors, cytokines and chemokines, which promote the regeneration of impaired liver [7].

The secretome or conditioned media is defined as the richly complex set of molecules secreted by stem cells or shed from their surface [8]. The idea of using stem cell secretome or conditioned media is based on the concept that the secretome is responsible for a considerable portion of the therapeutic potential of stem cells [9]. The MSC secretome contains a large number of growth factors and cytokines that are critical for the repair of injured tissues. Since paracrine factors released by stem cells can accumulate in the conditioned media (CM), it can be utilized as a cell-free therapy [10].

Epidermal growth factor (EGF) is a polypeptide produced in the submandibular glands, Brunner's glands and kidneys [11]. It stimulates growth of many cells including liver parenchymal cells both *in vivo a*nd *in vitro* [12] and it is expressed under normal physiological conditions in the adult liver, and gets markedly upregulated during the process of regeneration after liver injury or partial hepatectomy. The administration of EGF accelerates the proliferation of hepatocytes, regardless of what recombinant protein or gene therapy strategy was used [13, 14].

Therefore, the study was intended to investigate the potential of MSCs, conditioned media and EGF in promoting liver regeneration following partial hepatectomy in rat model.

Materials and Methods

Experimental Design

The study was designed after getting permission from the Institute Animal Ethics Committee (IAEC). Eighty clinically healthy adult Wistar rats (*Ratus norvegicus*), weighing 200-300 gm) of either sex were utilized for this study. Animals were procured from the Laboratory Animal Resources (LAR) section of the Institute. The animals were acclimatized to their environment 10 days prior to the study. The experimental animal models of partial hepatectomy in these animals were prepared and randomly divided into 4 groups, *viz.*, Groups A, B, C and D having 20 animals in each group. Animals of each group were given treatment according to Table 1.

The animals were sacrificed on 10^{th}, 20^{th}, 30^{th}, 40^{th} day and liver samples were collected for histopathology, immunohistochemistry and tracing of transplanted cells. The blood samples were also collected at similar time intervals; serum was separated from it and subjected to biochemical analysis. Clinico-physiological parameters were also recorded at the same time intervals.

Collection, isolation and cultivation of mesenchymal stem cells from rat

The bone marrow was obtained from femur and tibia of adult Wistar rat. After euthanizing the animal, the backside of the rats from the lumbar region to toes was shaved and prepared aseptically for dissection. The Skin incision was given on the lateral aspect of the thigh and both tibia and femur were exteriorized after removing the muscular and tendinous attachment. The femur and tibia were collected in a 50 ml tube containing Dulbecco's Phosphate Buffered Saline (DPBS) and antibiotics. The tubes were opened in the laminar flow under strict aseptic conditions. The metaphyseal region of the bones was cut and the bone marrow was flushed out by inserting a hypodermic needle into the medullary cavity using Dulbecco's Modified Eagles Media (DMEM) into a centrifuge tube. The bone marrow collected was then mixed with one volume of low glucose DMEM consisting of 10% fetal bovine serum (FBS), 1% penicillin-G, streptomycin sulphate and amphotericin B. The cell suspension was centrifuged at 980 rpm for 5 min to concentrate the cells. The cell pellet was resuspended in 5 ml of complete media and was layered over 5 ml of histopaque and then centrifuged at 2500 rpm for 30 min.

The nucleated cells were collected from the interface, washed with two volumes of DPBS and were then collected by centrifugation at 1500 rpm for 10 min. The cells were resuspended, counted and placed at 1×10^6 cells/cm^2 in T-75 flasks (Fig. 1). The adherent cells in the flask were maintained in Dulbecco's Modified Eagle's Medium-low glucose (DMEM-LG) containing 10% fetal bovine serum (FBS), 1% antibiotic mixture of 100 units/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen/Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After 4 days of primary culture, the non-adherent cells were removed by changing the medium. The medium was changed every 3 days thereafter until the flask showed confluency.

Table 1. Treatment protocol for animals in different groups

SI no.	Group	Number of animals (rat)	Treatment
1	A	20	Phosphate buffered saline (control)
2	B	20	Conditioned media
3	C	20	Rat bone marrow derived mesenchymal stem cells (r-BMSC)
4	D	20	Murine Epidermal growth factor (m-EGF)

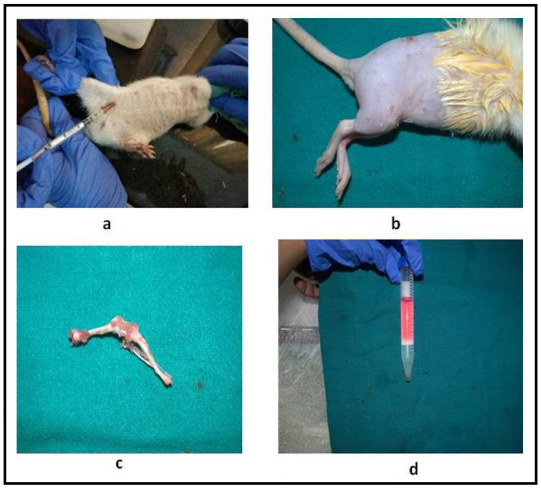


Fig. 1. Procedures for collection of bone marrow derived stem cells. (a) Intraperitoneal administration of anesthetics; (b) Animal preparation for bone marrow collection; (c) collection of femur & tibia; (d) density gradient separation of buffy coat.

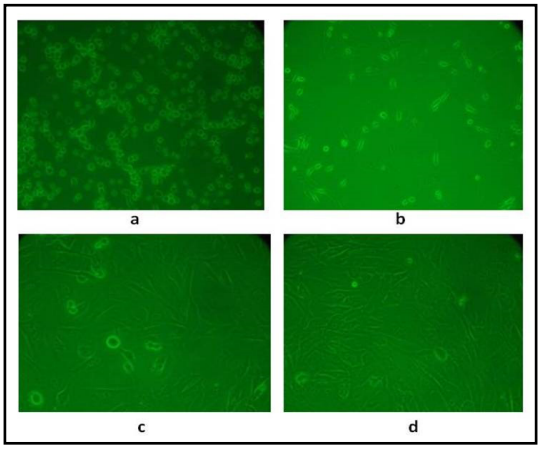


Fig. 2. Culturing of rat bone marrow derived mesenchymal stem cells. (a) P0 day 3; (b) P0 day 14; (C): P0 day 21; (d) Confluent P3.

After 18 days, the flask became 70-80% confluent and was passaged. Culture medium was removed, and cells were treated with Trypsin-EDTA solution (0.25% Trypsin and 1Mm EDTA (Gibco)) for 5 minutes at 37 °C to detach cells from the flasks. The Trypsin-EDTA activity was stopped by adding an equal volume of complete medium, and the medium with cells was collected in a centrifuge tube, centrifuged at 4 °C, 1500 rpm for 6 min. The supernatant was removed and the cell pellet was collected and placed in a T-75 flask. Third passage cells were used further for cell differentiation studies and *in vivo* experiments (Fig. 2).

Preparation of rat model of partial hepatectomy

The animals were anaesthetized with Midazolam @5mg/kg and Ketamine @50mg/kg intravenously. The cranio-ventral abdomen of the rats were shaved and sterilized with 5% povidoneiodine. The animals were restrained in dorsal recumbency. A midline incision was made from the xiphoid cartilage to umbilicus and cranial abdomen was exposed. The liver was exteriorized and three-knot technique was used at the base of the lateral and median lobe of the liver (divided into two lobes). Then respective lobes were resected after proper ligation. Bleeding was checked and abdomen wall and skin were sutured routinely in layers (Fig. 3). Antibiotic ceftriaxone 5mg/10ml of water and anti-inflammatory drugs butorphanol 0.5 mg/kg subcutaneous route was given for 5 days postoperatively. The animals were given stem cells (1.5×10^6, I/V), conditioned media (0.5ml, I/V), m-EGF (9µg, S/C) at three different intervals (Fig. 4).

Clinical parameters

Clinico-physiological parameters include rectal temperature was recorded on the day of surgery and for five days continuously post-operatively. Bodyweight was recorded at 10^{th}, 20^{th}, 30^{th}, and 40^{th} days postoperatively in all the animals.

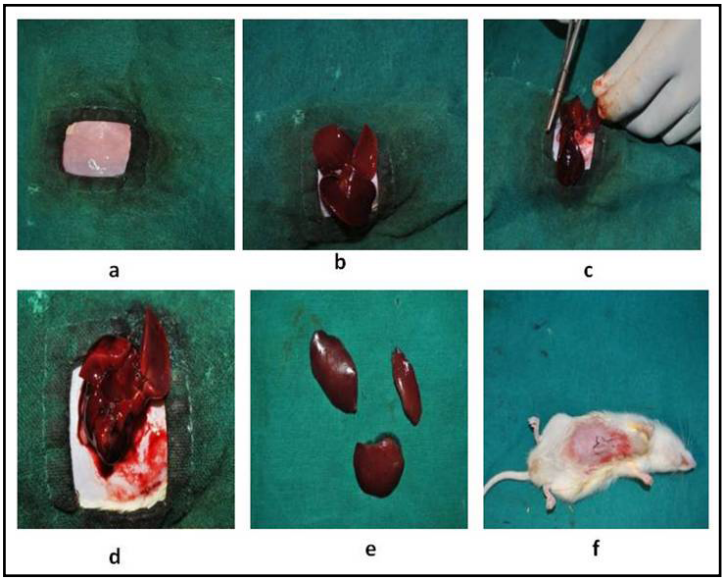


Fig. 3. Preparation of partial hepatectomy model. (a) Aseptic preparation of the surgical site; (b) Median and left later lobes were exposed; (c) Ligation of the median and left lateral lobes; (d) Applying sutures over the muscles; (e) Removed liver lobes; (f) Animal on dorsal recumbency after surgery.

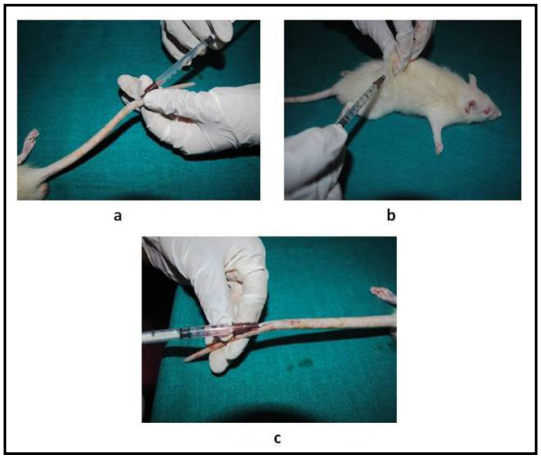


Fig. 4. Administration of r-BMSC (a), m-EGF (b) and conditioned media (c) in rats.

Biochemical parameters

Two ml of blood was collected from the orbital plexus of rats with capillary tubes at 10th, 20th, 30th and 40th days post operatively. One ml of blood was utilized for serum separation for estimation of alanine transaminases (ALT), aspirate transaminases (AST), alkaline phosphatase (ALP), albumin, total protein, bilirubin, gamma-glutamyl trnsferase (GGT) using standard commercially available kits. Blood glucose was estimated in the blood collected from the tip of the tail using a glucometer (On-Call-Plus).

Hematological parameters

One ml of blood was used for estimation of hemoglobin and packed cell volume (PCV) at 10th, 20th, 30th and 40th days' post-surgery.

Regeneration Coefficient

Regeneration coefficient was used for quantitative evaluation of the regeneration processes [15]. This Coefficient was calculated as follows:

$K= (P_1-P_2) / P_3$

Where P_1 is liver weight respective days after partial hepatectomy (measured directly), P_2 weight of the liver remaining after hepatectomy and P_3 weight of the removed liver (measured directly). P_2 is calculated by subtracting the initial weight of the liver (P_0) and the weight of the liver removed (P_3). The initial weight of

the liver was calculated based on the regression model and relationship between rat liver weight and body weight (y=0.0357x+1.7477), in this y is the liver weight and x is the body weight of the animal.

Enzyme linked immunosorbant assay

Matrix metalloproteinase-9 (MMP-9) was estimated by using enzyme linked immunosorbant assay (ELISA0 kit (Invitrogen Catalog # KHC3061) at 10^{th}, 20^{th}, 30^{th} and 40^{th} days postoperatively. Tissue inhibitors of matrix metalloproteinases-2 (TIMP-2) were estimated using rat TIMP-2, ELISA kit (Immuno Tag Catalog no: ITER0065) at 10^{th}, 20^{th}, 30^{th} and 40^{th} days postoperatively.

Histological observation

Liver tissues were collected on 10^{th}, 20^{th}, 30^{th} and 40^{th} day's interval for histological evaluation. Liver specimens were fixed in 10% neutral buffered formalin. The samples were then processed for paraffin embedding technique to get 5 micron thick paraffin section. The section was stained by Hematoxylin and Eosin stain (HE) to evaluate the regeneration process of liver tissue. Sections were also stained with Masson's Trichome for demonstration of collagen deposition in regenerated liver tissue at different intervals of an experiment.

Immunohistochemistry

Two hours before sacrifice, rats were administered 50 mg/kg 5-Bromo-2′-deoxyuridine (BrdU) intraperitoneal (Sigma-Aldrich, Catalog no: B5002). After being anaesthetized, liver tissues were removed and fixed with 10% neutral buffered formalin (NBF) for overnight, and then the tissues were shifted to fresh 10% NBF. Tissue sections 4 to 7μm thick were prepared. For immunohistochemical study Poly-L-Lysine (BOSTER Catalog no: AR0003) coated slides were used.

Tracing of transplanted cells

The confluence of BM-MSCs (third passage) when reached 60-70%, 4',6-diamidino-2-phenylindole (DAPI; 1 μg/ml) (Invitrogen Catalog # D1306) was added to the medium and cells were cultured for 12 h. After incubation, cells were washed with PBS 6 times and the cell sample was examined by fluorescence microscopy. The DAPI stained BM-MSCs were trypsinized counted and about 1.5×10^{6} cells were injected intravenously through the tail vein. For identifying DAPI+ BM-MSCs in the liver, the liver was harvested on day 9 after surgery and frozen sections (5μ thickness) were prepared and observed under the fluorescence microscope.

Statistical analysis

The data were analyzed by using the Statistical Program for Social Sciences (SPSS 20 IBM). One way ANOVA was done to compare the means at different time intervals among different groups. Repeated measures ANOVA were performed for comparing the mean values between different time intervals within a group. A value of $P<0.05$ (*) was considered to be statistically significant and at times $P<0.01$ (**) was considered to be statistically significant. All the graphs were prepared in Graph Pad Prism (Version 5).

Results

Clinical parameters

Rectal temperature. Rectal temperature was recorded for 6 days as it is depicted in Fig. 5. The rectal temperature was measured on the day of surgery before anaesthesia and then the temperature was recorded for 5 consecutive days postoperatively.

Bodyweight. The bodyweight of the animals in different groups at various time intervals is depicted in Fig. 6. The bodyweight of all the animals was measured before surgery and then subsequently recorded on 10^{th}, 20^{th}, 30^{th} and 40^{th} days postoperatively. The bodyweight of the animals didn't show significant variation within groups.

Fig. 5. Mean ± SE of rectal temperature of different groups at various time intervals.

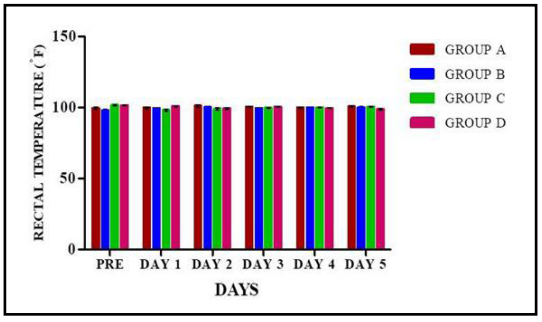


Fig. 6. Mean ± SE of body weight of different treatment groups at various time intervals.

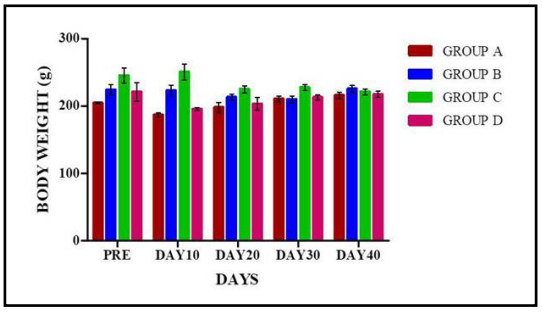


Table 2. Mean ± SE of ALT (U/L) of different treatment groups at various time intervals. *Mean value differ significantly at P< 0.05 within the group; **Mean value differ extremely significantly at P<0.01 within the group

Groups	Pre	Day 10	Day 20	Day 30	Day 40
A	53.9±0.49	64.39±0.59**	61.67±1.87*	58.78±0.59*	58.28±1.48
B	53.49±1.22	56.43±0.8	53.72±1.02	56.63±2.79	56.43±1.89
C	55.59±1.34	58.54±2.55	52.92±0.81	55.23±1.75	55.72±2.1
D	52.72±0.28	56.92±2.84	53±0.9	59.33±2.5**	55.18±1.64

Table 3. Mean ± SE of AST (U/L) of different treatment groups at various time intervals. *Mean value differ significantly at P< 0.05 within the group; **Mean value differ extremely significantly at P<0.01 within the group

Groups	Pre	Day 10	Day 20	Day 30	Day 40
A	100.3±0.64	156.72±8.64**	136.37±4.63**	137.52±5.49*	141.03±5.36**
B	98.97±1.24	103.91±1.18	105.98±3.55	113.89±7.26	123.08±15.34
C	97.81±4.41	118.43±7.37	126.04±11.01**	142.56±5.37**	138.18±4.12**
D	100.42±0.57	120.66±15.53	126.8±7**	135.91±6.2*	106.5±2.52

Haemato-Biochemical parameters

Alanine transaminase. The Alanine transaminase (ALT) values in different treatment groups are depicted in the Table 2. It was observed that ALT values did not significantly differ between groups at any time intervals. In group A there was significant (P<0.05) increase in ALT values on day 10, day 20 and day 30 compared to their basal values. In group D there was a significant (P<0.05) increase in ALT value on day 30 compared to its basal values. However, in other groups, no significant change was observed.

Aspartate transaminase. The Aspartate transaminase (AST) values in different treatment groups are depicted in the Table 3. The AST values did not significantly vary between the different groups during various time intervals. However, it was observed that AST value increased significantly (P<0.05) at all periods compared to their basal values in some of the groups. In group C, the level of AST was elevated on day 20, day 30 and day 40 while in group D on day 20 and day 30. However, the rest of the groups did not show significant variations within themselves at various periods.

Alkaline phosphatase. The Alkaline phosphatase (ALP) values in different groups at various time intervals are depicted in Table 4. It was observed that ALP values did not vary significantly between groups at various time intervals. However, there was significant ($P<0.05$) increase in ALP values in group A on day 10 and day 40 compared to basal values. In group C there was a significant ($P<0.05$) increase in ALP values on day 10, day 20 and day 40. In group D there was a significant increase ($P<0.05$) noticed at all periods.

Albumin. The albumin concentration in different treatment groups at various time intervals are depicted in Fig. 7. It was observed that albumin concentration decreased significantly ($P<0.05$) in group A compared with their base values on day 10. However, in other groups, it didn't show significant variation between different time intervals within each group.

Total protein. The total protein concentration in different treatment groups at various time intervals are depicted in Fig. 8. The protein concentration did not show significant variation within each group between different time intervals except in groups A and D where there was significant ($P<0.05$) decline in concentration on day 10 compared to their base values. In group A there was a significant decline ($P<0.05$) in total protein concentration on day 20 and day 40 compared to its base values.

Table 4. Mean ± SE of ALP (U/L) of different treatment groups at various time intervals. *Mean value differ significantly at P< 0.05 within the group; **Mean value differ extremely significantly at P<0.01 within the group

Groups	Pre	Day 10	Day 20	Day 30	Day 40
A	150.42±4.71	222.58±18.64*	194.73±24.28	170.24±9.78	199.78±16.25*
B	169.97±18.61	213.95±23.94	140.02±4.8	159.02±14.69	185.28±12.18
C	121.75±8.14	198.95±26.84*	191.66±10.06**	156.08±9.57	194.17±11.55**
D	132.25±11.09	236.56±14.84**	186.9±6.21*	185.12±8.65*	179.77±9.28*

Fig. 7. Mean ± SE of albumin (g/dl) of different treatment groups at various time intervals.

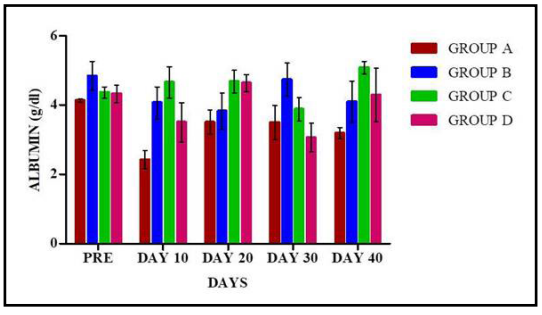
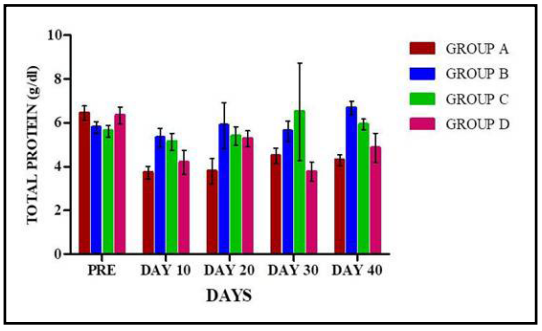


Fig. 8. Mean ± SE of total protein (g/dl) of different groups at various time intervals.



Bilirubin. The bilirubin concentration in different treatment groups at various time intervals are depicted in Table 5. In this study, it was observed that bilirubin concentration did not vary significantly within different groups at various time intervals except group A. In group A, the bilirubin concentration was increased significantly (P<0.05) on day 20 and day 40. It was recorded that the bilirubin concentration was significantly (P<0.05) higher in group A compared to groups B and D.

Gamma glutamyl transferase. The Gamma glutamyl transferase *(*GGT) level in serum of GGT level in serum of animals from different treatment groups is displayed in Table 6. It was revealed that GGT value in all groups did not vary significantly within groups at any time intervals except in group D. The GGT value in group D was significantly (P<0.05) higher on day 20 when compared to all other groups.

Blood glucose. The blood glucose values in different treatment groups at various time intervals were recorded and displayed in Fig. 9. It was observed that blood glucose was significantly (P<0.05) higher in group A on the day of surgery compared to groups D, while in other groups it didn't show any significant difference. The blood glucose value in all groups didn't show significant variation within groups except in group A where the values significantly (P<0.05) declined on day 10, day 30, day 40.

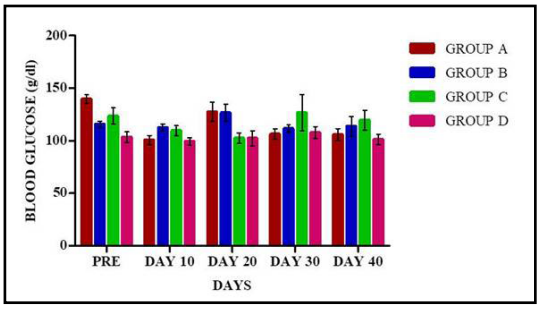
Table 5. Mean ± SE of bilirubin (mg/dl) of different treatment groups at various time intervals. *Mean value differ significantly at P< 0.05 within the group; **Mean value differ extremely significantly at P<0.01 within the group; Different superscript indicate mean value differ significantly at P<0.05 between group

Groups	Pre	Day 10	Day 20	Day 30	Day 40
A	0.14±0.01	0.18±0.03	0.26±0.04^{a**}	0.24±0.03	0.23±0.01^{a*}
B	0.18±0.02	0.19±0.02	0.15±0.01^{bcd}	0.26±0.03	0.19±0.03^{ab}
C	0.16±0.01	0.18±0.05	0.17±0^{abcd}	0.22±0.08	0.2±0.01^{ab}
D	0.16±0.02	0.17±0.05	0.16±0.01^{bcd}	0.18±0.01	0.2±0.04^{ab}

Table 6. Mean ± SE of gamma glutamyl transferase (U/L) of different treatment groups at various time intervals. *Mean value differ significantly at P< 0.05 within the group; **Mean value differ extremely significantly at P<0.01 within the group; Different superscript indicate mean value differ significantly at P<0.05 between group

Groups	Pre	Day 10	Day 20	Day 30	Day 40
A	1.31±0.11	5.65±0.58	2.54±0.22^{a}	3.21±0.31	1.09±0.32^{a}
B	1.24±0.19	4.48±0.28	2.28±0.34^{a}	2.08±0.35	1.94±0.28^{ab}
C	1.36±0.16	6.55±0.35	1.88±0.48^{a}	2.97±0.93	1.06±0.52^{a}
D	1.17±0.25	5.62±0.42	3.38±0.39^{b**}	3.99±0.79	2.55±1.26^{ab}

Fig. 9. Mean ± SE of blood glucose of different treatment groups at various time intervals.



Hemoglobin. The hemoglobin of animals from different treatment groups was recorded on the day of surgery and days 10, 20, 30 and 40 postoperatively and is depicted in Fig. 10. The hemoglobin concentration did not significantly vary among different groups at various time intervals. There was decrease in hemoglobin concentration on 10th day after surgery in all groups, but significant decline ($P<0.05$) was noticed in group A compared to its base values.

Packed cell volume

The packed cell volume (%) in different treatment groups are depicted in Fig. 11. The packed cell volume in animals in different treatment groups at various time intervals were recorded on the day of surgery and days 10, 20, 30 and 40 postoperatively. There was no significant variation in the packed cell volume among different groups at various time intervals. The PCV showed a decline in the values on 10th day post-surgery in all the groups but in group A, it was a significant ($P<0.05$) decrease compared to their base values.

Regeneration Coefficient

The regeneration coefficients were calculated in all the groups and are displayed in Table 7. It was observed that regeneration coefficient did not show significant change within the groups at various time intervals, however, there was a non-significant increase in group C.

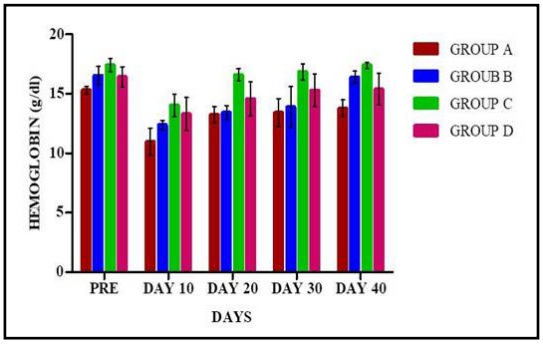


Fig. 10. Mean ± SE of hemoglobin of different treatment groups at various time intervals.

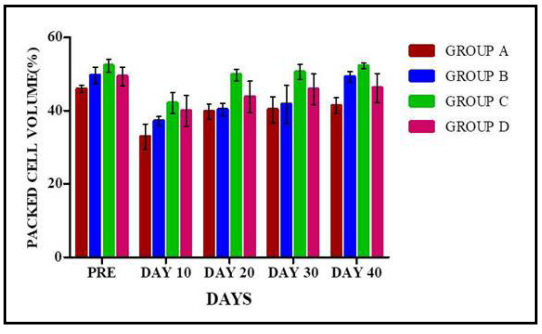


Fig. 11. Mean ± SE of Packed cell volume (%) of different treatment groups at various time intervals.

Table 7. Mean ± SE of regeneration coefficient of different treatment groups at various time intervals. Different superscript indicate mean value differ significantly at $P<0.05$ between group

Groups	Day 10	Day 20	Day 30	Day 40
A	0.63±0.02^{a}	0.68±0.05^{a}	0.66±0.02^{a}	0.69±0.01^{a}
B	0.72±0.02^{ab}	0.71±0.03^{ab}	0.71±0.02^{ab}	0.74±0.02^{ab}
C	0.8±0.07^{ab}	0.81±0.06^{ab}	0.82±0.02^{abc}	0.86±0.03^{abc}
D	0.73±0.06^{ab}	0.75±0.04^{ab}	0.75±0.06^{abc}	0.76±0.06^{abc}

Matrix metalloproteinase

The Matrix metalloproteinase (MMP-9) level in different treatment groups at various time intervals are displayed in Table 8. The matrix metalloproteinase level had significantly (P<0.05) increased in all the groups at 10, 20, 30 and 40 postoperative days compared to their base values except in group A and group B; where no significant change was observed on day 10. It was observed that MMP-9 value was significantly (P<0.05) lower in groups A and B than all other groups on day 10. The MMP-9 value was significantly (P<0.05) lesser in group A followed by group B at all time intervals except on day 30. The MMP-9 value was significantly (P<0.05) higher in group C followed by that in groups D, B, A in the descending order.

Tissue inhibitors of matrix metalloproteinase

The concentration of tissue inhibitors of matrix metalloproteinase (TIMP-2) in different treatment groups at various intervals are displayed in Table 9. In group A there was significant (P<0.05) increase in TIMP-2 concentration on day 10, day 20 and day 40 compared to its base values whereas in all other groups there was no significant change. It was observed that the values for TIMP-2 were significantly (P<0.05) higher in groups A and B compared to all other groups. The TIMP-2 concentration was significantly higher on day 20 in group A and group B compared to all other groups. On the 40th day the TIMP-2 concentration was significantly highest (P<0.05) in groups A, followed by group B and group C. The TIMP-2 value was significantly (P<0.05) lowest in group D.

Colour digital imaging

In this study, colour photographs of the liver from different treatment groups at various time intervals were taken. This digital imaging was done to assess the gross changes in the size, shape and other changes in the liver. In group A, Fig. 12a depicts the colour images of the liver taken on days 10, 20, 30 and 40 postoperatively. It can be grossly observed that the size of the liver as such did not in increase much on day 10 while subsequently hypertrophy was observed in other lobes but the liver was comparatively paler in appearance. On the 40th day, the size of the liver comparatively increased concerning its day 10 size. In group B, Fig. 12b depicts the colour images of the liver taken on days 10, 20, 30 and 40 after treatment with conditioned media. It was observed that grossly the liver size in this group did not vary significantly compare to control group (A) on day 10, but subsequently on day 30 and 40 enlargement and hypertrophy in the liver lobes was observed. In group C, Fig. 12c displays

Table 8. Mean ± SE of MMP-9(ng/ml) of different treatment groups at various time intervals. *Mean value differ significantly at P< 0.05 within the group; **Mean value differ extremely significantly at P<0.01 within the group; Different superscript indicate mean value differ significantly at P<0.05 between group

Groups	Pre	Day 10	Day 20	Day 30	Day 40
A	2.66±0.04^{ab}	2.75±0.09^{a}	3.44±0.13^{a}*	5.6±0.02^{ab}**	4.89±0.03a**
B	2.95±0.06^{a}	2.23±0.02^{a}	4.55±0.08^{b}**	5.18±0.15^{a}**	6.14±0.1^{b}**
C	2.77±0.02^{ab}	4.34±0.05^{b}**	3.64±0.02^{a}*	5.61±0.11^{ab}**	7.8±0.19^{cde}**
D	2.43±0.14^{ab}	5.51±0.47^{c}**	4.1±0.28^{abc}**	5.36±0.03^{ab}**	7.48±0.19^{d}**

Table 9. Mean ± SE of TIMP-2(ng/ml) of different treatment groups at various time intervals. *Mean value differ significantly at P< 0.05 within the group; **Mean value differ extremely significantly at P<0.01 within the group; Different superscript indicate mean value differ significantly at P<0.05 between group

Groups	Pre	Day 10	Day 20	Day 30	Day 40
A	49.73±7.76	86.61±1.18^{a}*	86.77±1.33^{a}*	75.36±2.3^{a}	86.64±11.76^{a}*
B	63.06±4.73	71.22±1.79^{a}	71.37±1.94^{a}	64.66±1.76^{ab}	67.03±3.06^{ab}
C	50.88±7.21	42.18±4.27^{bc}	42.33±4.43^{b}	47.84±6.24^{bde}	60.42±7.37^{abc}
D	57.73±5.46	36.66±4.88^{bc}	36.81±4.73^{b}	29.54±6.0^{de}	57.97±4.55^{abcd}

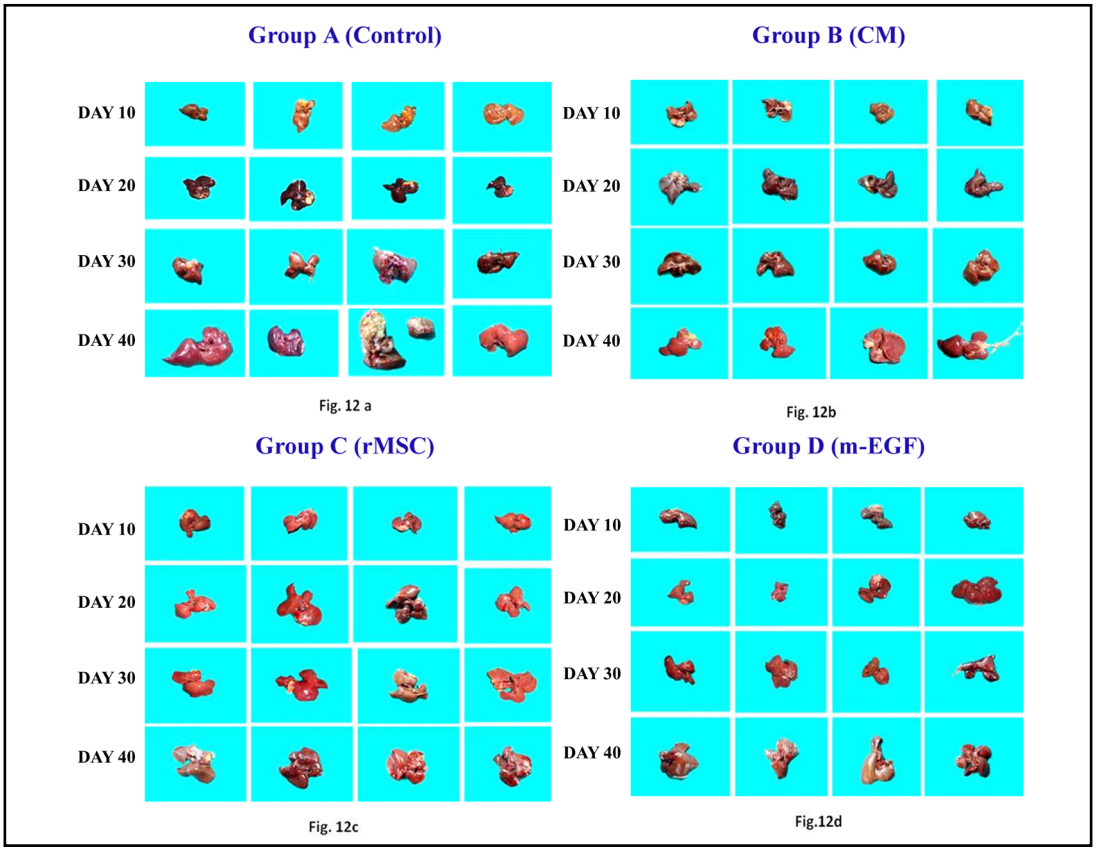


Fig. 12. (a-d) Colour digital imaging of groups A, B, C and D.

the colour images of liver taken on days 10, 20, 30 and 40 postoperatively after treatment with bone marrow derived mesenchymal stem cells. It was noticed that there was a gross enlargement in the size of the liver from day 10 onwards, there was hypertrophy and the colour and texture of the liver was quite similar to its normal appearance. In group D, Fig. 12d depicts the colour images of the liver taken on day 10, 20, 30 and 40 postoperatively after treatment with epidermal growth factor. It was observed that on day 10 there was not much enlargement in the size of liver but was better than group A and group B, subsequently, there was enlargement and hypertrophy in the size of liver noticed on days 30 and 40.

Histopathological observations

The histopathological examination of the liver samples collected in various treatment groups at different time interval was performed. Histopathological evaluation of rat liver at day 40 after partial hepatectomy is presented in Table 10. Various histopathological alterations were photographed using an upright microscope (Olympus, BX3, Japan). In group A, there was bile duct proliferation, lymphocyte infiltration and fibrous tissue proliferation on day 10 (Fig. 12a). On day 20, there was fibroblast proliferation, bile pigment deposition, lymphocyte reaction and coagulative necrosis. On day 30, it was observed that there was fibroblast proliferation, neovascularization, few newly formed hepatocytes. On day 40, few newly formed hepatocytes could be seen; enlarged cells with hyperchromatic nuclei and vacuolations were also observed. In group B, there was bile duct hyperplasia, bile pigment deposition, fibrous tissue proliferation on day 10 (Fig. 13b). On day 20, no distinct sinusoidal arrangements and fibrous tissues extension from portal triad were seen. On day 30, Kupffer cells were seen in the regenerated area, newly formed hepatocytes could be seen. On day 40, there was bile duct proliferation along with more number of Kupffer cells in the regenerated area, no distinct boundary of hepatocytes. In group C, bile duct proliferation and newly

formed hepatocytes could be visualized in the regenerated area on day 10 (Fig. 13c). On day 20, newly formed hepatocytes, dividing hepatocytes and lymphocyte infiltration could be visualized. On day 30, newly formed hepatocytes and bile pigment deposition were noticed.

Table 10. Histopathological evaluation for rat hepatic tissues at day 40. 0=none; 1=minimal; 2=mild; 3=moderate; 4=severe

Histopathological parameters	Group A	Group B	Group C	Group D
Increased number of biliary ducts	2	2	2	2
Inflammatory cell infiltration	3	3	1	2
Degenerative cellular changes	4	3	2	2
Focal hepatocyte necrosis	3	3	2	2
Alteration in the sinusoidal area	3	2	2	2
Total	15	13	9	10

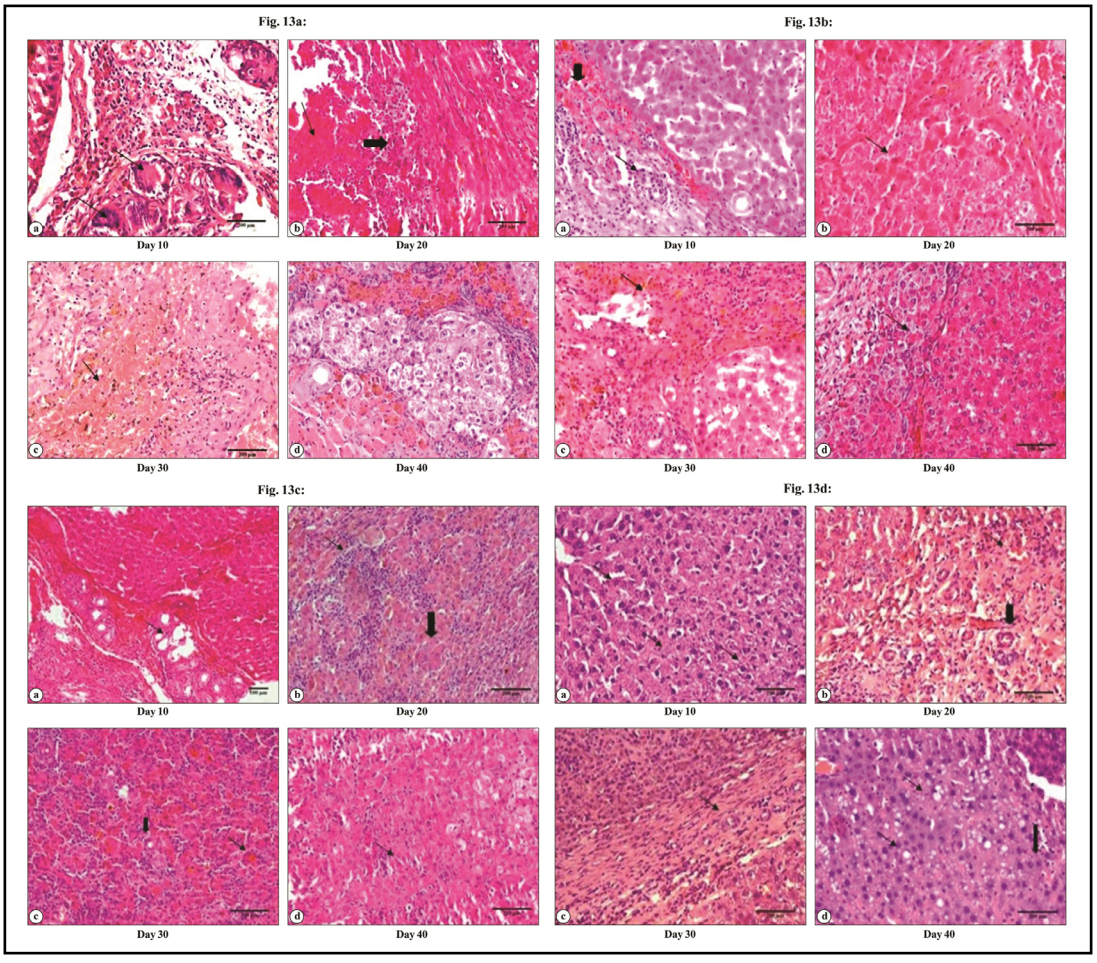


Fig. 13. Histopathological observations (H& E stained, 20 x) of groups A, B, C and D on days 10, 20, 30 and 40. 13a: group A (a) bile duct proliferation(arrows) on day 10; (b) Areas of coagulative necrosis (thin arrow), lymphocyte infiltration (thick arrow) on day 20; (c) bile pigment deposition (arrow), fibroblast proliferation on day 30; (d) Enlarged cells with hyperchromatic nuclei and vacoulations on day 40. 13b: group B (a) bile duct proliferation (thin arrow), bile pigment deposition(thick arrow) on day 10; (b) sinusoidal arrangement not distinct ,newly formed hepatocytes (arrow) on day 20; (c) bile pigment deposition (arrow), Kupffer cells increased in number on day 30; (d) Newly formed hepatocytes with no distinct boundary on day 40. 13c: group C (a) bile duct proliferation (thin arrow on day 10; (b) Lymphocyte infiltration (thin arrow), newly formed hepatocytes (thick arrow) on day 20; (c) bile pigment deposition (arrow), newly formed hepatocytes (thick arrow) on day 30; (d) Sinusoidal arrangement not distinct on day 40 (arrow). 13d: group D (a) More number of dividing hepatocytes (thin arrow) on day 10; (b) N eovascularization (thin arrow) on day 20, bile duct proliferation (thick arrow) on day 20; (c) Bile duct proliferation (arrow) on day 30; (d) Fatty changes (thin arrows) neovascularization (thick arrows) on day 40.

On day 40, abundant dividing hepatocytes was found in the regenerated area, however, sinusoidal arrangement was not properly formed. In group D, dividing nuclei inside the hepatocytes and more number of Kupffer cells could be seen on day 10 (Fig 13d). On day 20, enlarged nuclei inside the hepatocytes, neovascularization and newly formed bile ducts could be seen. On day 30, newly formed hepatocytes and bile duct proliferation could be noticed. On day 40, more neovascularization, fatty changes and newly formed hepatocytes were noticed.

Masson's Trichrome

Masson's trichrome staining was performed to evaluate the fibrotic changes in the liver. Masson's Trichrome staining in normal liver demonstrated hexagons with the delicate lining of connective tissue. In group A (Fig. 14a) there was collagen deposition in between the newly formed hepatocytes and around the proliferated bile duct which subsequently increased from day 10 onwards upto day 40. In group B (Fig. 14b) there was fibroblast proliferation in between the hepatocytes the broadening of fibroplasia subsequently got reduced and more organized. In group C (Fig. 14c) the fibrous tissue proliferation was very limited around the bile ducts and at the junction of regeneration, which was later reduced and was scanty. In group D (Fig. 14d) the fibrous tissue was noticed on day 10 which later got reduced and was more organized on subsequent days. Scoring based on Masson's Trichrome staining of rat hepatic tissue after partial hepatectomy is depicted in Table 11.

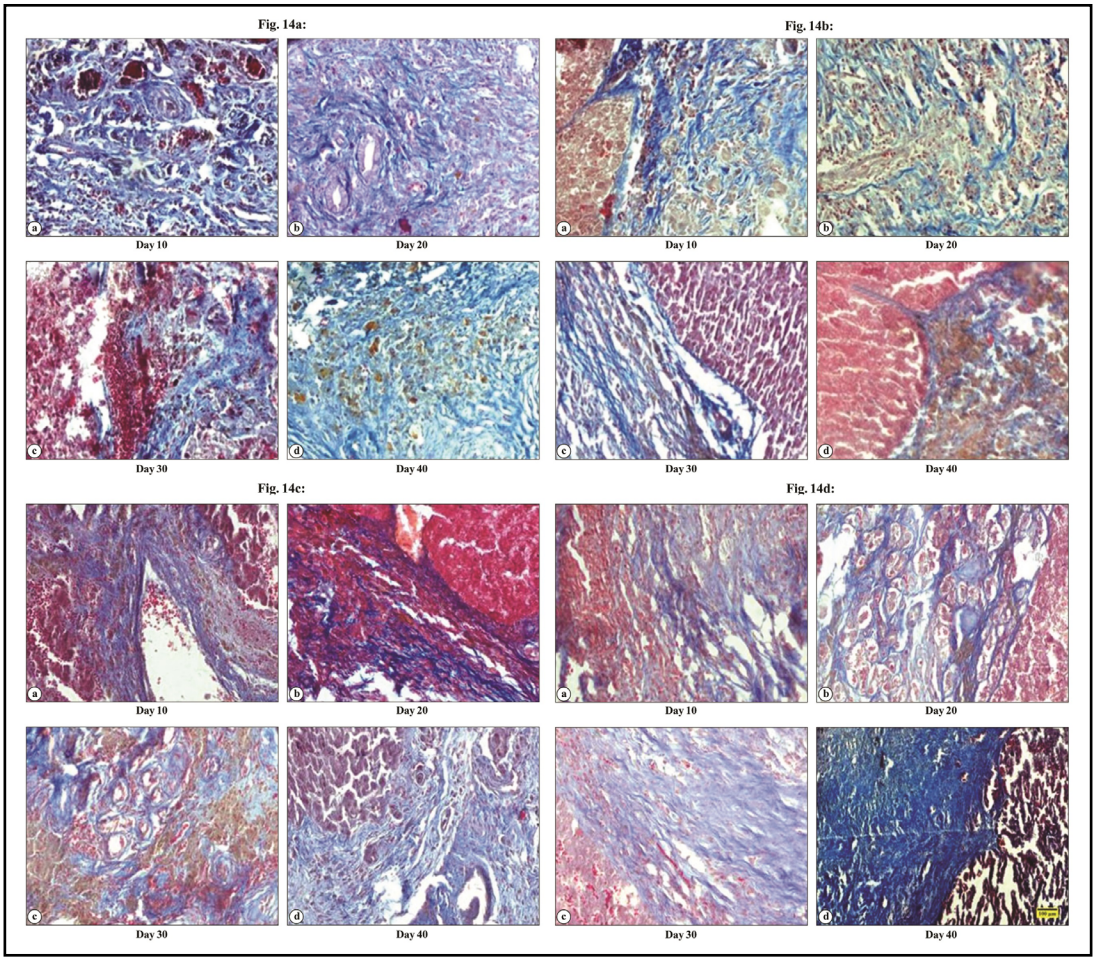


Fig. 14. (a-d): Masson's Trichome staining (20x) of groups A, B, C and D at different time intervals (d10; 20; 30, 40). 13a: group A; 13b: group B, 13c: group C; 13d: group D.

Table 11. Scoring based on Masson's Trichrome staining of rat hepatic tissues. 0=none; 1=minimal; 2=mild; 3=moderate; 4=severe

Groups	Day 10	Day 20	Day 30	Day 40
A	3	4	4	4
B	2	3	3	3
C	2	3	2	2
D	2	3	3	3

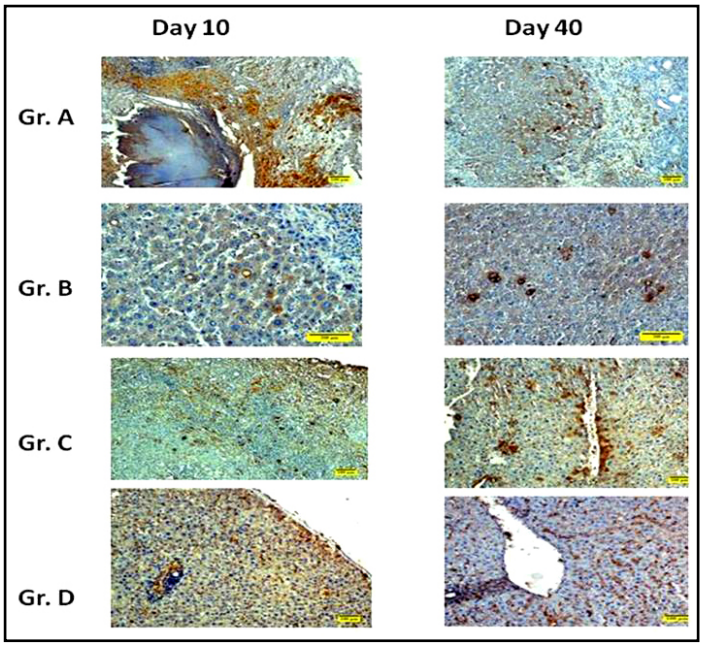


Fig. 15. Immunohistochemical quantification of BrdU uptake proliferating hepatocytes in groups A, B, C, D at day 10 and 40.

Immunohistochemistry

Immunohistochemistry was done to evaluate the uptake of BrdU by the regenerated cells. The Fig. 15 depicts the images of BrdU staining in different treatment groups on day 10 and day 40. The BrdU stained cells were less in group A at period 10, 40. In group B, the BrdU stained cells were less but higher than group A at both time intervals. The BrdU stained cells were higher in number in groups in which stem cells were given (group C). The Table 12 showing the number of cells that has taken at days 10 and at days 40 in different groups.

Table 12. Number of cells that have taken BrdU at days 10 and 40 in different treatment groups

Groups	Day 10	Day 40
A	16	23
B	19	28
C	26	41
D	23	39

Tracing of transplanted cells

The bone marrow-derived mesenchymal stem cells (third passage cells) were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 µg/ml). For this purpose, DAPI was added in the media and the cells were cultured for 12 h. The cells were observed under a fluorescent microscope and it was observed that the nucleus of the stem cells was showing fluorescence (Fig. 16). These DAPI stained stem cells were trypsinized and injected intravenously through a tail vein in partially hepatectomized rats. After 9 days of therapy, the animal was sacrificed and the liver was collected. Frozen section of the liver tissue was prepared (5µ) and observed under the microscope. DAPI labelled stem cells were observed in the liver section.

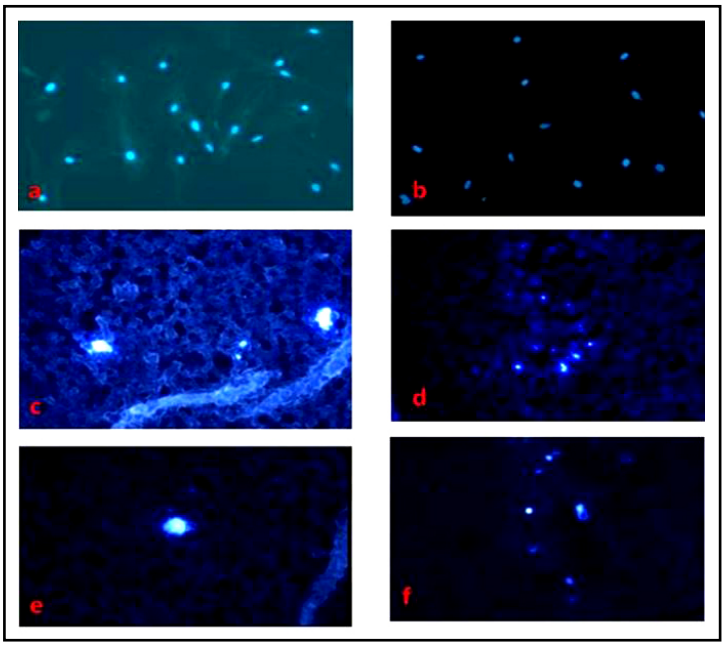


Fig. 16. Tracing of transplanted r-BMSC; a & b (20x) showing r-BMSC labeled with DAPI, c, d, e (20x) & f (10x) showing DAPI labeled stem cells in liver sections 9 days post therapy.

Discussion

Animal models, particularly the rat model, are commonly used to investigate new aspects of liver regeneration. Liver resection has been refined and used many times over after Higgins and Anderson [16] presented a method for standardized partial hepatectomy. The term partial hepatectomy (PHx) means a surgical resection of one or more liver segments. The word is derived from the Ancient Greek, *hepat*: "liver" and *ectomy*: "to cut out". The rat liver is having four lobes median, left, right and caudate; all lobes except left are further divided into two or more parts. The classical 70% hepatectomy is the most popular and extensively used model than other models include 90, 95 and 97 percent. In the 90% hepatectomy the right lobes, median lobe and the left lateral lobe are resected. In the 95% hepatectomy model, the anterior caudate is also removed, while, in the 97% hepatectomy, the anterior caudate and posterior caudate are removed, and only the paracaval portion remains [17]. Several studies have been conducted for investigating the initial stages of liver regeneration that too for a short period of time. In this study, the left lateral and the median lobes were resected out and it was approximately 68-70% hepatectomy. This study was targeted for later stages of regeneration and for a longer time compared to previous trials. The procedure was standardized well for creating an animal model for this study.

The rectal temperature showed a significant decline in group B when compared to group C and group D on the day of surgery while other groups didn't show any significant variations among each other. The experiments in group B was performed during winter, the temperature of the extremely cold environment might be a reason for the reduction in the body temperature before surgery in this group. Despite of all this, all the animals had the rectal temperature in the normal range (96.62-99.50°F) throughout the observation period. In this study, there was a significant decline in the body weight in groups A and D while groups B and C had higher body weight than the above-mentioned groups but they didn't show any significant variations among themselves. After partial hepatectomy pain and inflammation might have contributed to the reduced feed consumption and subsequent reduction in the body weight. The animal loses weight to supply metabolites drawn from other tissues but subsequently renders to the loss and gains back its weight [18]. In this study in group A, there was a gradual decrease in the bodyweight of the animal after partial hepatectomy but it regained back its weight after 40 days. Animals in the other experimental groups more or less maintained their body weight throughout the study period without much fluctuation.

In all the treatment groups there was increase in ALT value post hepatectomy but the change was not significant except in group A where there was a significant increase in serum ALT values. Elevated circulating ALT is a known marker of liver damage, specifically of hepatocytes. Mesenchymal stem cells derived conditioned media maintains sinusoidal epithelial cell and hepatocytes integrity [19] that may be the reason for not much fluctuation in ALT level in the groups in which conditioned media was given. The MSC treatment ameliorated the increase in serum transaminase levels, which serves as the most sensitive marker for clinical and experimental hepatic I/R injury evaluation [20]. The subcutaneous EGF administration was very capable of increasing hepatic proliferation [21] that restores its function and prevents fluctuation in ALT. The normal level of ALT is around 52U/L in Wistar rats. In this study, there was a significant increase in the value of AST in groups A, C, D at different time intervals while in other groups it didn't show significant variations. The increased level of AST in serum is indicating damage in the liver. It is recognized that serum transaminases are very sensitive in the demonstration of hepatocyte damage and independent from etiological factors; their values remain at high levels as far as persistence of liver damage [22]. As a cytoplasmic and mitochondrial enzyme, AST is found in many organs apart from liver including heart, skeletal muscle, and kidney and brain tissues [23]. In this study, there was a significant increase in AST at all periods in group A compared to its basal values owing to the persisting hepatic damage, since the animal was given only PBS postoperatively. However, the AST levels were maintained in other groups within the normal range of (74-143U/L).

In this study, there was a significant increase in ALP values in groups A, C, D while in other groups it didn't show any significant variations. Alkaline phosphatase (ALP) is indicative of cholestasis. Normally alkaline phosphatase occurs in the cells of bile canaliculi. Partial hepatectomy causes increased production of bile canalicular cells [24] leading to an increased level of enzyme in the serum. The ALP level was significantly higher in group A compared to its basal value which can be due to intrahepatic cholestasis due to injury and subsequent delay in the healing process.

In this study, there was a significant decrease in albumin level at day 10 in group A with respect to its base value and this decline was also observed in other intervals. However, in other groups, it didn't show any significant variation between themselves. The normal albumin levels in rat's ranges between 3.8-4.8 g/dl. After partial hepatectomy, the remnant liver increases its albumin production to make up the normal protein value. In this study in group A, the albumin production has declined significantly on day 10, since no therapy was given, the hepatic parenchymal damage was not able to compensate for the loss in albumin production. The findings were in accordance with the results in the previous study [25]. The total protein in group A declined significantly at all periods and it was least on day 40 compared to all other groups while other groups maintained more or less normal range of protein and didn't show any significant variations among themselves. The total protein is expected to fall postoperatively due to hepatic insufficiency of the remaining liver. Since the liver is the major site of synthesis of plasma proteins and albumin. The total protein significantly declined in group A throughout the treatment period indicates insufficient hepatic function. In groups in which EGF, r-BMSC and conditioned media were given showed great improvement in hepatic function and total protein level were more or less maintained in the normal range of 6.8-7.5 mg/dl. The results of this study were following the previous findings [26].

In group A, there was an increase in the value of total bilirubin at all periods concerning to its basal value, while in other groups there was an increase in the level of bilirubin but it got subsequently reduced during the study period. Bilirubin content of blood and liver is one of the potent indicators of RBC destruction and physiological state of the liver. The normal range of bilirubin varies from 0.05-0.18 mg/dl in rats. In group A, there was a comparatively significant increase in the bilirubin value on day 20 and 40. It can be due to an increased load of cell destruction and accumulation of bilirubin on substantially reduced liver cell mass [26]. The level of γGT increased on day 10 in all the groups but then it declined subsequently.

However, the γGT was significantly elevated in group D at day 20, whereas in other groups didn't show significant variations among themselves. The gamma glutamyl transferase level in rat's ranges from 0-1 U/L. γGT is concentrated in the cell membranes instead of cytosol. However, a variety of substances is known to exert regulatory effects on liver regeneration and is therefore potentially involved with the induction of GGT. These substances include hydrocortisone [27], triiodothyronine [28], insulin, glucagon, epidermal growth factor [29], parathyroid hormone [30], and catecholamines [31]. This might be the reason for elevated level of γGT on day 20 in group D in which EGF was given alone as a treatment in this study.

There was a significant decrease ($P<0.05$) in the value of blood glucose in group A on days 10, 30, 40 while in other groups it didn't show any significant variations. A minor decrease in circulating blood glucose after partial hepatectomy has been recorded and it is associated with a compensatory decrease in insulin and an increase in glucagon secretion [32]. The fall in blood glucose reflects the acute decrease in functional hepatic parenchymal mass, which recovers sufficiently to restore normoglycaemia. There was a significant decline in the hemoglobin, PCV may be due to blood loss after surgery and subsequently reduced heme synthesis from the reduced liver mass.

Khnychenko et al. [15] have used regeneration coefficient for evaluation of regeneration after partial hepatectomy in rats after therapy with potassium orotate. In this study, we evaluated the regeneration in various groups using regeneration coefficient. In this study group, A had significantly lesser regeneration coefficient.

It was observed that there was a gradual trend of increase in the value of MMP-9 in all groups after surgery and therapy. Among all, group A had the least MMP-9 value and group C had the highest MMP-9 followed by groups D, B in the decreasing order. In contrary TIMP-2 level was significantly higher in group A followed by in groups B, C, D in the descending order. The infusion of BMCs elevated the levels of matrix metalloproteinase (MMP-2, MMP-9 and MMP-14) reduced liver fibrosis and improved the survival rate [33, 34]. During liver fibrosis, the relative ratio of MMPs to TIMPs is decreased. Thus fibrosis may be progressive because of a decrease in matrix degradation. Activated stellate cells in fibrotic liver show a low expression of MMPs (except MMP-2) and a high expression of TIMPs and respond to TGF-β1 with further upregulation of TIMP, reflecting the MMPs/TIMPs expression pattern detected in fibrotic livers [35]. In this study on day 40, the MMP-9 level was least in group A while TIMP-2 level was highest in this group. The results are following the reasons described above. In this study grossly in group A, the liver size was comparatively lesser, the normal texture of the liver was not attained and it was somewhat paler. When the liver was sectioned it was tougher. In group B the liver size was better than group A but lesser than groups C and D in which comparatively better liver size and the texture was observed. Twenty-four hours after partial hepatectomy, active cellular replication process starts and continues till the liver reaches its baseline weight. Within the first 10 days, major regenerative changes occur and this process is completed within 4 or 5 weeks. Excised lobes do not assume their previous configuration. Regeneration more frequently proceeds as the formation of new lobules and enlargement of the remaining lobules [36]. All liver cells divide and involved in the regeneration process [37].

Hepatocytes which constitute 80% of the liver mass and 60% of the number of hepatic cells, most rapidly induce cellular regeneration cycle. These cellular changes occur within minutes [38]. Histopathological evaluation was made semi-quantitatively based on inflammatory cell adhesion and/or migration, bile duct proliferation, presence and intensity of cellular degenerations, alterations in sinusoidal area. Based on this scoring system [39] maximum degenerative changes were observed in group A, in this group foamy degeneration, necrotic foci, fibrosis, collagen deposition, infiltration of neutrophils were evident. It was closely followed by group B in which infiltration of neutrophils and other degenerative changes were there, though few bile duct proliferation were noticed in one section. Group C, D showed almost similar kind of histopathological changes characterized by bile duct proliferation, negligible degenerative changes, mitotic figures or dividing hepatocytes. Masson's Trichrome staining demonstrated the collagen deposition in the liver at various

periods and collagen deposition was highest in group A followed by that in groups B, D, C in decreasing order. Maximum DNA synthesis is seen in hepatocytes within 24 h after a liver injury or partial hepatectomy. Hepatocytes are followed by ductular epithelial cells, Kupffer cells, stellate cells and sinusoidal endothelial cells in order of decreasing rates of regeneration [40]. It has also been suggested that bone marrow cells could differentiate into hepatocytes after severe liver injury. When the severe liver injury occurs, bone marrow stem cells quickly migrate to the liver and differentiate into hepatocytes [41]. In this study, the injected BM-MSCs could migrate to the damaged liver and might differentiate into hepatocytes to promote liver regeneration after partial hepatectomy. Histopathological examination of liver tissue showed that MSC has an significant antifibrotic effect as evidenced by the decrease in liver collagen stained with Masson Trichrome [42]. MSC produce trophic factors that will directly act on stellate cells and induce their apoptosis, they inhibit the proliferation of stellate cells and also inhibit collagen synthesis from these stellate cells, all these contribute to the anti-fibrotic effect of r-BMSC [7].

Kimura et al. [43] recently reported that exogenous EGF and glycyrrhizin significantly stimulated both liver regeneration and recovery of function *in vivo* in response to partial (70%) hepatectomy, and also proposed that both of them possibly work via stimulation of EGF receptor. Glaneman et al. [21] reported that an increase in mitotic figures in histopathology of the liver after treatment with EGF.

MSC-CM reduces neutrophils infiltration and Kupffer cell activation, thus downregulating the expression of inflammatory cytokines [44]. MSC-CM treated rats showed no signs of disseminated inflammation, although minor periportal infiltration with oedema and fibrin deposition consistent with tissue repair was also observed in previous studies [45]. Massive centrilobular necrosis, central vein dilation, ballooning degeneration and inflammatory cellular infiltration of the liver are associated with liver damage evidenced with histological findings [46] was also evidenced in the control group (A) of this study where no stem cells or growth factor was given.

In this study maximum, BrdU uptake was observed in group C on day 40 followed by groups D, B and A in the decreasing order. Least uptake was observed in group A, indicating less hepatic cell proliferation. In the groups in which epidermal growth factor was administered there was greater uptake of BrdU in the newly formed vessel walls showing greater neovascularization. The results of this study were consistent with the results of the previous study [25].

The blue fluorescent DAPI (4',6-diamidino-2-phenylindole) nucleic acid stain preferentially binds to double-stranded DNA to form a stable fluorescent complex that fluoresces approximately 20 times greater than DAPI alone. DAPI exhibits a preference for repetitive A-T base pairs in the minor groove [47, 48]. DAPI is a popular nuclear counter stain for use in multicolor fluorescent techniques, mRNA *in situ* hybridization, and *in vivo* cell tracking experiments. In this study, the DAPI-labeled stem cells were injected through tail vein after partial hepatectomy. After 9 days of the therapy, the animal was euthanized and liver was collected. Frozen sections of the liver observed under the fluorescent microscope. The fluorescent dye DAPI binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity. DAPI, once added to tissue culture cells, is rapidly taken up into cellular DNA, yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence. In this study, the DAPI-labeled stem cells were detected in the frozen section of the liver, which is following the previous studies [25].

Conclusion

In conclusion, biochemical, gross, histopathological and immunohistochemical results revealed better, organized and optimum liver regeneration in groups C and D. Mild hepatogenesis was found in group B, whereas very negligible/less hepatogenesis was found in group A.

Acknowledgements

The authors wish to thank Dr K. P. Singh, Joint-Director, CADRAD, ICAR-IVRI, Izatnagar (UP), India for his technical assistance in evaluation of histological sections. The authors also wish to thank the Director, ICAR-Indian Veterinary Research Institute, Izatnagar, India and Head, Division of Surgery, Indian Veterinary Research Institute for providing necessary facilities for this study.

Author Contributions

DM and SKM designed the study, analyzed the data and prepare the manuscript; DM, SS, SP, KE, BBV, SDS and RKR performed the experiments; NK, SC and DM performed haemato-biochemical estimation; DM performed histopathological evaluation; JH edited the manuscript.

Funding

No external funding agencies supported this research work.

Statement of Ethics

Protocols for this study were approved by the Institute Animal Ethics Committee for Animal Care and Animal Experimentation.

Disclosure Statement

The authors declare that no conflicts of interest exist.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P: Estimating the world cancer burden. Globocan 2000. Int J Cancer 2001;94:153-156.
2 Kanat O, Gewirtz A, Kemeny N: What is the potential role of hepatic arterial infusion chemotherapy in the current armamentorium against colorectal cancer. J Gastrointest Oncol 2012;2:130-138.
3 Helling TS: Liver failure following partial hepatectomy. HPB (Oxford) 2006;8:165-174.
4 Houlihan DD. Houlihan, P, Newsome N: Critical review of clinical trials of bone marrow stem cells in liver disease. Gastroenterology 2008;135:438-450.
5 Körbling M, Estrov Z: Adult stem cells for tissue repair-a new therapeutic concept, New Eng J Med 2003;349:570-582.
6 Eom YW, Kim G, Baik SK: Mesenchymal stem cell therapy for cirrhosis: present and future perspective. World J Gastroenterol 2015a;21:10253.
7 Eom YW, Shim KY, Baik SK: Mesenchymal stem cell therapy for liver fibrosis. Korean J Intern Med 2015;30:580-589.
8 Makridakis M, Roubelakis MG, Lahou A: Stem cells: insights into the secretome. Biochim Biophys Acta 2013;1834:2380-2384.
9 Yoon BS, Moon JH, Jun EK, Kim J, Maeng I, Kim JS, Lee JH, Baik CS, Kim A, Cho KS, Lee JH: Secretory profiles and wound healing effects of human amniotic fluid-derived mesenchymal stem cells. Stem Cells Dev 2009;19:887-902.
10 Lee SK, Lee SC, Kim A: A novel cell-free strategy for promoting mouse liver regeneration: utilization of a conditioned medium from adipose-derived stem cells. Hepatol Int 2015;9:310-320.
11 Olsen PS, Nexø E, Poulsen SS, Hansen HF, Kirkegaard P: Renal origin of rat urinary epidermal growth factor. Regul Pept 1984;10:37-45.
12 Jansing R, Samsonoff WAS: Effect of epidermal growth factor on cultured adult rat hepatocytes. Tissue Cell 1984;16:157-166.

13 Kiso S, Kawata S, Tamura S, Inui Y, Yoshida Y, Sawai Y, Umeki S, Ito N, Yamada A, Miyagawa JI, Higashiyama S: Liver regeneration in heparin-binding EGF-like growth factor transgenic mice after partial hepatectomy. Gastroenterology 2003;124:701-707.
14 Khai NC, Takahashi T, Ushikoshi H, Nagano S, Yuge K, Esaki M, Kawai T, Goto K, Murofushi Y, Fujiwara T, Fujiwara H: *In vivo* hepatic HB-EGF gene transduction inhibits Fas-induced liver injury and induces liver regeneration in mice: a comparative study to HGF. J Hepatol 2006;44:1046-1054.
15 Khnychenko LK, Stepanov II, Sapronov NS: Regression analysis of relationship between liver weight and body weight after partial hepatectomy in rats. Bull Exp Biol Med 2006;141:269-271.
16 Higgins GM, Anderson RM: Experimental pathology of the liver: restoration of the liver of the white rat following partial surgical removal. Arch Pathol Lab Med 1931;12:186-202.
17 Martins PN, Theruvath TP, Neuhaus P: Rodent models of partial hepatectomies. Liver Int 2008;28:3-11.
18 Sánchez QE, Soberón G, Palacios O, Lee E, Kuri M: Changes in effective enzyme concentration in the growing rat liver. II. Liver regeneration after partial hepatectomy. J Biol Chem 1961;236:1607-1610.
19 Chen YX, Zeng ZC, Sun J, Zeng HY, Huang Y, Zhang ZY: Mesenchymal stem cell-conditioned medium prevents radiation-induced liver injury by inhibiting inflammation and protecting sinusoidal endothelial cells. J Radiat Res 2015;56:700-708.
20 Kanazawa H, Fujimoto Y, Teratani T, Iwasaki J, Kasahara N, Negishi K, Tsuruyama T, Uemoto S, Kobayashi E: Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. PloS One 2011;6:191-195.
21 Glanemann M, Shi B, El-Zidy N, Gaebelein G, Kronbach Z, Neuhaus P, Nussler AK: Subcutaneous administration of epidermal growth factor: A true treatment option in case of postoperative liver failure. Int J Surg 2009;7:200-205.
22 El-Ashmawy IM, El-Nahas AF, Salama OM: Protective effect of volatile oil, alcoholic and aqueous extracts of Origanum majorana on lead acetate toxicity in mice Basic Clin Pharmacol. Toxicol 2005;97:238-243.
23 Delva E, Camus Y, Nordlinger B, Hannoun L, Parc R, Deriaz H, Lienhart A, Huguet C: Vascular occlusions for liver resections. Operative management and tolerance to hepatic ischemia: 142 cases. Ann Surg 1989;209:211-218.
24 Allalouf DS, Schwarzman H, Levinsky N, Feller J, Hart S, Zoher R, Menache R: Lactate dehydrogenase and alkaline phosphates isoenzymes in regenerating liver. Res Exp Med 1986;186:103-107.
25 Li DL, He XH, Zhang SA, Fang J, Chen FS, Fan JJ: Bone marrow derived mesenchymal stem cells promote hepatic regeneration after partial hepatectomy in rats. Pathobiology 2013;80:228-234.
26 Siddique M: Comparison of liver function tests after 2/3 hepatectomy and liver biopsy in experimental rats. J Med Sci 2004;4:318-322.
27 Henderson IC, Fischel RE, Loeb JN: Suppression of liver DMA synthesis by hydrocortisone. Endocrinology 1971;88:1471-1476.
28 Short J, Wedmore R, Kilbert L, Zemel R: Triiodothyronine: on its rote as a specific hepatomitogen. Cytobios 1980;28:165-177.
29 McGowan JA, Strain AJ, Bucher NLR: DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium: effects of epidermal growth factor, insulin, glucagon and cyclic AMP. J Cell Physiol 1981;708:353-363.
30 Rixon RH, MacManus JP, Whitfield JF: The control of liver regeneration by calcitonin, parathyroid hormones, and 1a,25-dihydroxychotecalciferol. Mol Cell Endocrinol 1979;75:79-89.
31 Moriey CGD, Royse VL: Adrenergic agents as possible regulators of liver regeneration. Int J Biochem 1981;73:969-973.
32 Strecker W, Silz S, Salem A, Ruhenstroth-Bauer G: Metabolic changes in the serum of partially hepatectomized rats. Horm Metab Res 1980;12:604-608.
33 Fang B, Shi M, Liao L, Yang S, Liu Y, Zhao RC: Systemic infusion of FLK1+ mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice. Transplantation 2004;78:83-88.
34 Sakaida I, Terai S, Nishina H, Okita K: Development of cell therapy using autologous bone marrow cells for liver cirrhosis. Med Mol Morphol 2005;38:197-202.
35 Takahara T, Furui K, Yata Y, Jin B, Zhang LP, Nambu S, Sato H, Seiki M, Watanabe A: Dual expression of matrix metalloproteinase-2 and membrane-type 1-matrix metalloproteinase in fibrotic human livers. Hepatology 1997;26:1521-1529.

36 Değerli U, Bozfakıoğlu Y (eds): Cerrahi Gastroenterology, 5th ed. Istanbul, Basım, Nobel Tıp Kitabevleri, 2000, pp 194-208.
37 Widmann JJ, Fahimi HD: Proliferation of mononuclear phagocytes (Kupffer cells) and endothelial cells in regenerating rat liver. A light and electron microscopic cytochemical study. Am J Pathol 1975;80:349.
38 Laurent S, Otsuka M, De Saeger C, Maiter D, Lambotte L, Horsmans Y: Expression of presumed specific early and late factors associated with liver regeneration in different rat surgical models. Lab Invest 2001;81:1299-1307.
39 Cetinkunar S, Tokgoz S, Bilgin BC, Erdem H, Aktimur R, Can S, Erol HS, Isgoren A, Sozen S, Polat Y: The effect of silymarin on hepatic regeneration after partial hepatectomy: is silymarin effective in hepatic regeneration. Int J Clin Exp Med 2015;8:2572-2578.
40 Tanaka Y, Mak KM, Lieber CS: Immunohistochemical detection of proliferating lipocytes in regenerating rat liver. J Pathol 1990;160:129-134.
41 Tokcaer-Keskin Z, Akar AR, Ayaloglu-Butun F, Terzioglu-Kara E, Durdu S, Ozyurda U, Ugur M, Akcali KC: Timing of induction of cardiomyocyte differentiation for *in vitro* cultured mesenchymal stem cells: a perspective for emergencies. Can J Physiol Pharmacol 2009;87:143-150.
42 Aziz MA, Atta HM, Mahfouz S, Fouad HH, Roshdy NK, Ahmed HH, Rashed LA, Sabry D, Hassouna AA, Hasan M: Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis. Clin Biochem 2007;40:893-899.
43 Kimura M, Moro T, Motegi H, Maruyama H, Sekine M. Okamoto H, Inoue H, Sato T, Ogihara M: *In vivo* glycyrrhizin accelerates liver regeneration and rapidly lowers serum transaminase activities in 70% partially hepatectomized rats. Eur J Pharmacol 2008;579:357-364.
44 Du Z, Wei C, Cheng K, Han B, Yan J, Zhang M, Peng C, Liu Y: Mesenchymal stem cell-conditioned medium reduces liver injury and enhances regeneration in reduced-size rat liver transplantation. J Surg Res 2013;183:907-915.
45 Parekkadan B, Van Poll D, Suganuma K, Carter E A, Berthiaume F, Tilles AW, Yarmush ML: Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PloS one 2007;2:941.
46 Plaa GL, Hewitt WR: Quantitative evaluation of indices of hepatotoxicity; in Plaa GL, Hewitt WR (eds): Toxicology of the Liver. New York, Raven Press, 1982, pp 103-120.
47 Hajduk SL: Demonstration of kinetoplast DNA in dyskinetoplastic strains of Trypanosoma equiperdum. Science 1976;191:858-859.
48 Kania J, Fanning TG: Use of a Sequence-Specific DNA-Binding Ligan to Probe the Environments of EcoRI Restriction Endonuclease Cleavage Sites. Eur J Biochem 1976;67:367-371.