Original article

Phase-dependent expression profiling and quantification of several growth factors in liver regeneration after partial hepatectomy

Adeela Hussain[1], Gibran Ali[1,2], Muhammad Awais Afzal[1], Asima Tayyeb[3],Shehla J Akram[4] and Javed Akram[2*]

[1] Center of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan

[2] Institute of Regenerative Medicine, Physiology and Cell Biology Department, University of Health Sciences, Lahore, Pakistan

[3] School of Biological Sciences, University of the Punjab, Lahore, Pakistan

[4] Akram Medical Complex, Lahore, Pakistan

*jakramaimc@gmail.com

Abstract:

Growth factors are the potential operational members which control different phases of liver regeneration. Different growth factors have expression regulation in the whole process relating to different phases of liver regeneration. **Objective:** To assess the expression regulation of different growth factors and cytokines involved in liver regeneration in a phase-dependent manner. **Methods:** Blood and liver samples were collected and analyzed on 1st, 3rd, 5th, 7th and 14th postoperative days after 50% Partia hepatectomy (PHx). **Results:** Steady increase of liver regeneration rate was recorded from 90.8% (1st day) to 97.9% (7th day). Liver function tests further confirmed the steady liver recovery in PHx mice. Several growth factors such as HGF and VEGF exhibited an up-regulation till 5th day and later gradual decrease till 14th day compared to control mice. Albumin, CK18 and CK19 showed sequential expression increase from 1st to 14th day compared to AFP and HNF-4α upregulated until 5th and 1st day, respectively. Quantification of these growth factors further confirm our results. **Conclusions:** Conclusively, these results highlight a phase-dependent regulation and role of growth factors in liver regeneration and recovery.

Keywords: Liver regeneration, Partial hepatectomy, Growth factors, Hepatic markers, Gene expression analysis, Liver function tests

Introduction:

Liver is one of the intriguing organs with incredible regenerative competence. The wide array of liver functions has been shielded by its phenomenal regenerative capacity. The unusual regenerative power of the liver is a logical adaptation by organisms as it works as the core detoxifying organ of the body and more likely to be injured by the ingested toxins [1, 2]. Studying the mechanisms involved in natural liver regeneration could be useful in understanding and treating diseased liver. Loss of liver mass can be stimulated by hepatotoxic chemicals or by surgical methods. Mostly, liver regeneration is studied by a surgical procedure in which half of the liver mass is removed in rodents known as partial hepatectomy (PHx) introduced first by Anderson in 1931 [3]. The rodent liver is a multi-lobe structure and amputation of any of its lobes can easily be done without causing any tissue damage to the rest of lobes. They grow to refurbish an aggregate equivalent to the mass of

original lobes. This process completes within 5-7 days after hepatectomy [4].

Repopulation of the liver can be attained through one of two mechanisms, as self-replication of individual cell types or as trans-differentiation from facultative stem cells or liver progenitor stem cells [5]. After two-third PHx in mice, hepatocytes are the first type of liver cells to enter DNA synthesis. Hepatocytes in its normal state rarely divide but capable of repopulating the liver in case of deficit hepatic mass. Their behavior illustrate that they can go through one or two rounds of cell proliferation during liver regeneration [6]. Liver regeneration has been initiated by cytokines signals mediated by norepinephrine, Notch/Jagged, bile acids and IL-6 in first 4 hours [7]. Transcription factors are the proteins, produced at early stage, which bind to the specific recognition sites of the genes to initiate and enhance their transactivation [8]. HNF-4α is the member of liver enriched hepatocyte nuclear transcription factor family and play key role in the initiation of the liver regeneration process after partial hepatectomy. It binds to the hepatocyte specific DNA regulatory region providing synergistic transcriptional activation [9].

HGF, VEGF, SDF1α, TGF-α, EGF acts not only on cell proliferation but also on morphogenesis, angiogenesis, cell motility, differentiation and cell survival [10-13]. The regeneration process is histologically well described, but the genes that orchestrate liver regeneration have been only partially characterized. There is no easy scalable experimental approach available to correlate the expression of growth factors to the initiation and proliferation of regenerative events going on in liver after partial hepatectomy. The complexity of these signaling pathways initiating and terminating the regenerative process of liver have provided paradigms for regenerative medicine.

Liver can regenerate after PHx of mouse and tissue and serum samples were collected at different time points between 1st day to 14th day. The expression profiling of different growth factors; HGF, VEGF and SDF1α was performed. The role of transcription factor HNF-4α in triggering liver regeneration was also described. Functionality of regenerated liver was assessed by liver biochemical assays. Data showed for the first time a comparison of expression of different growth factors genes in different phases of liver regeneration which is further confirmed by hepatic genes and liver function tests. Further research in this direction will provide useful information that can be applied clinically for treating liver diseases and *in vitro* organogenesis.

Methods:

Animals:

Studies were performed on C57BL/6 mice aged 8 weeks and weighing 20-25g kept under controlled temperature and light conditions with excess food and water *ad libitum*. All animal handling and experimental procedures were in accordance with the guidelines of the Committee of Animal Care, National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan. Animals were divided into five groups based on sampling days (1st, 3rd, 5th, 7th and 14th).

Preparation of Hepatectomy models and Sampling:

Anesthesia was induced and maintained with ketamine (88 mg/Kg of body weight) and xylazine (14 mg/Kg of body weight) in all groups. 50% partial hepatectomy was performed with median laparotomy in hepatectomy groups. Groups C1 to C14 (control groups) underwent an identical surgical procedure without partial hepatectomy. On the 1st, 3rd, 5th, 7th and 14th postoperative days, animals were sacrificed and regenerated livers were removed, weighed, and processed for further analysis. Blood was taken directly from heart to perform liver function tests.

Body weight measurement:

Weights of all animals in each experimental group were measured before surgery and at the time of sampling. The acquired data was expressed as the percentage rate of liver

regeneration and calculated according to the following equation: Rate of regeneration (%) = Average weight of mice at sampling day (A)/ Average weight of mice before surgery (B) × 100.

Gene Expression Analysis:

RNA from liver tissue of all experimental groups was extracted using TRIZOL reagent (Invitrogen, Inc. USA). cDNA was synthesized using cDNA synthesis kit (Fermentas). Gene specific primers (Table 1) were designed using online software Primer3. Gene expression analysis was done by reverse transcriptase PCR (RT-PCR) and quantitative analysis (qRTPCR) performed on PikoReal 96 (Thermo Scientific, USA) in control and hepatectomy groups (n = 3) using MAXIMA SYBRR Green qPCR Master Mix (Thermo Scientific, USA). The relative gene expression was then analyzed using SDS software (ABI). β-actin and GAPDH was used as an internal control.

Immunohistochemical Analysis:

Paraffin embedded tissue sections from control and hepatectomy groups were examined for hepatic markers After deparaffinization and rehydration steps, sections were fixed with 4% PFA for 15 min, followed by washing with PBS thrice for 5 min. Blocking was done with 10% normal donkey serum for 45 min at RT. Tissue sections were incubated with respective primary antibodies (anti-albumin and anti-CK18, dilution 1:50) at 4°C overnight in a humidified chamber. Tissues were again washed thrice and incubated with respective fluorescence labeled secondary antibodies for 1 hour at RT. After washing three times with PBS, DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma Aldrich, USA) staining was done for 15 minutes at room temperature. After washing with PBS thrice, sections were mounted with Vectashield mounting medium and observed under Olympus BX61 (Olympus, Japan) microscope. At least 20 images were randomly taken for each group.

Western Blot Analysis:

Total protein was extracted from 50mg liver tissue by adding 500µl RIPA buffer (Sigma-Aldrich, USA) containing protease inhibitor cocktail (Calbiochem, USA). Bradford assay was performed for protein quantification using BioRad protein assay dye reagent concentrate according to manufacturer's instructions (Bradford, 1976). 50µg of each protein extract in sample buffer was loaded into SDS-PAGE gel and electrophoresed. Separated proteins were transferred to nitrocellulose membrane by using a semi dry transblot system for 1 h. The membrane was blocked with 5% skim milk for 2 hours at RT. After blocking, membrane was incubated with respective primary antibodies: VEGF (1:100), SDF1α (1:200), HGF (1:100) and βactin (1:200) overnight at 4°C under shaking conditions. Next day, blots were washed three times for 5 min each with 1X TBST. Blots were incubated with respective HRP conjugated secondary antibodies for 1 h at RT followed by three times washing for 10 min each with 1X TBST. The proteins on the blot were then detected by chromogen substrate DAB (MP BioMedicals, France).

Serum isolation:

Blood samples from ventricle of mice heart were collected in 1ml BD syringes at the respective day of sampling and transferred into 1.5ml centrifuge tubes. The samples were kept at room temperature for 2-3 hours followed by centrifugation at 10,000xg for 20 min. The clear supernatant was transferred in new 1.5ml centrifuge tube. The serum was stored at -70°C until further use.

Estimation of Growth factors by ELISA

ELISA was carried out to measure the concentrations of growth factors (HGF, VEGF and SDF1α,) and hepatic marker (Albumin). 96 well ELISA plates were coated with diluted serum samples along with coating buffer (1X PBS) in triplicate sets. Standards were also coated for each growth factor separately. The plates were incubated at 4°C overnight. After 24 hours, plates were washed with washing buffer

and blocked with 5% skim milk for 1 h at 37°C. After washing again thrice, 100µl primary antibodies: HGF (1:400), VEGF (1:100), SDF1α (1:500) and Albumin (1:500) were added in previously serum coated plates overnight at 4°C. After washing three times, 100 µl of the HRP conjugated secondary antibody (1:1000) were added to each well and incubated for 1 hour at 37°C. After washing, 100µl of chromogenic substrate (TMB reagent; Sigma Aldrich, USA) was added. After sufficient development of blue color, 100µl of stop buffer was added. Reading was taken at 450nm with ELISA plate reader (BioTek, USA).

Liver Function Tests:

To access the functionality of liver after partial hepatectomy, biochemical assays of bilirubin, ALAT and Alkaline phosphatase were performed from isolated serum. Commercially available kits were used according to the manufacturer's protocol (DiaSys, Germany).

Statistical Analysis:

All data were presented as mean ± SD. Significant differences between the experimental groups were determined by using two-way ANOVA using Graph-Pad prism software**.** Statistical significance was considered at $p \leq 0.05$.

Results:

Increase in rate of liver regeneration:

The serial changes in liver regeneration rate in hepatectomy models were statistically increased on day 1, 3, 5 and 7 with 90.8%, 92.1%, 95.2% and 97.9% while on day 14 rate of regeneration slightly reduced to 96.8%. In control models, no change was observed (Figure 1).

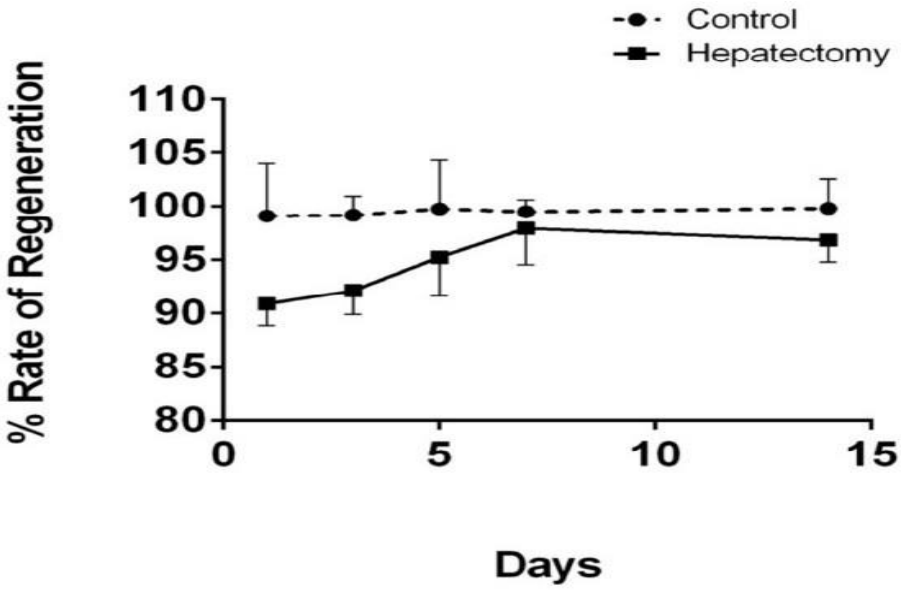

Figure 1: % Rate of liver regeneration after PHx. Rate of regeneration increased in H1, H3, H5 and H7 groups

Gene expression analysis after PHx:

The gene expression of AFP was increased at day 1, 3 and 5 and then was decreased at day 7and day 14 as it is a marker of early liver development, while, its expression in control groups remained lowered and constant. The expression of Albumin was progressively increased after PHx. Contrary to this; CK18 expression is almost same in all experimental groups. While, CK19 expression was started increasing at day 5 after PHx and remained high until day 14. The expression of HNF-4α was up

regulated only at day 1 and then down regulated in all other groups. HGF expression was remained up-regulated until day 7 and was decreased at day 14. Similarly, VEGF was remained up-regulated until day 5 but, start decreasing at day 7. SDF1α expression was started increasing at day5 after PHx and remained high until day 14 (Figure 2). The expression of hepatic genes, AFP expression was decreased after 5th day as it is a marker of early liver development. Albumin, CK18 and CK19 was gradually increased as liver regeneration progressed. HNF-4α expression was increased initially but then decreased in the later stages of liver regeneration. The expression of HGF and VEGF was increased in the early stages but later decreased. There is minor expression of SDF1α in control and hepatectomy groups. GAPDH was used as internal control.

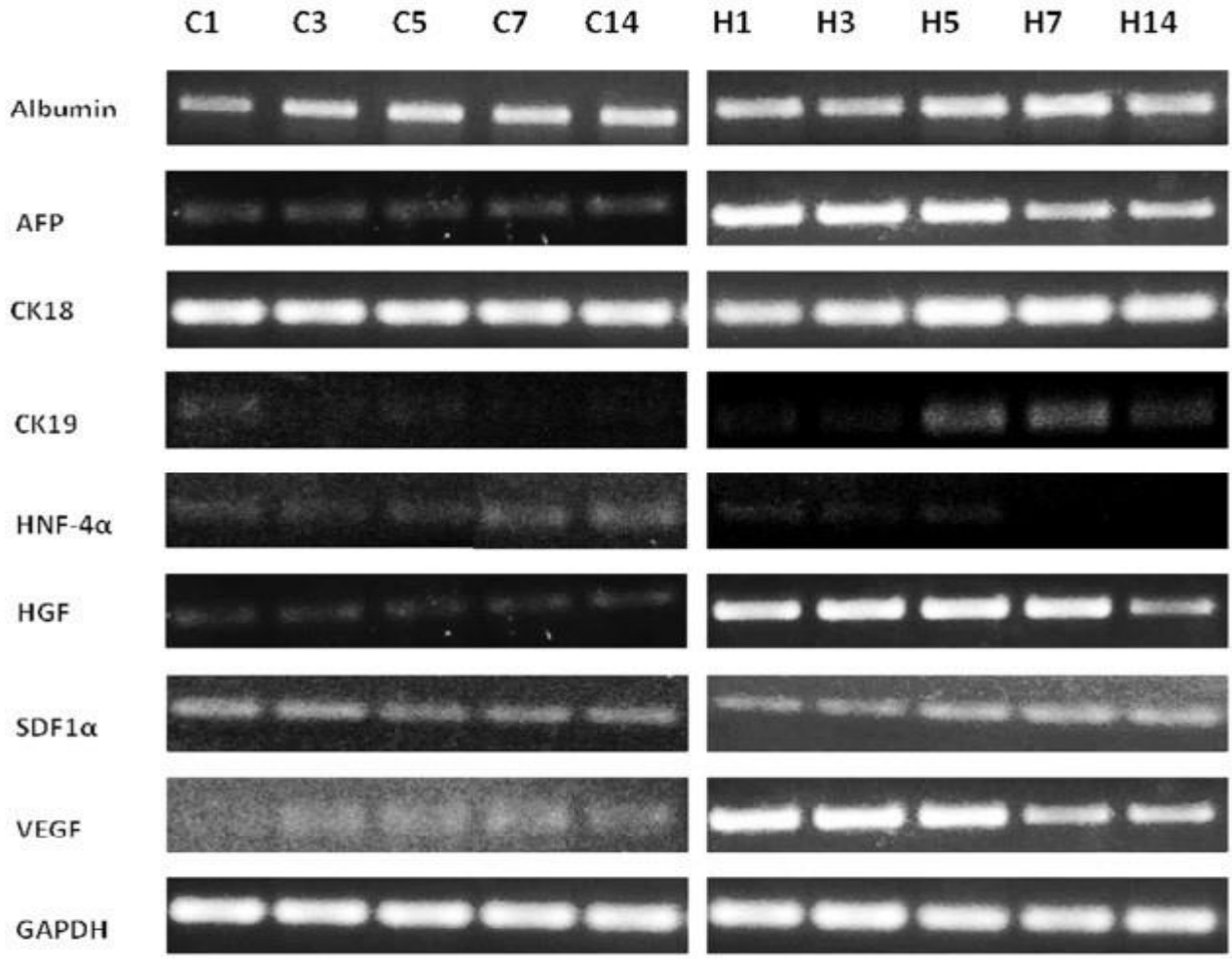

Figure 2: Expression of hepatic and growth factors genes after PH

Real Time PCR data also confirmed that AFP expression was increased significantly to 4.66 ± 0.20 folds and then starts decreasing from day 3 to day 14 (3.47 ± 0.41, 1.63 ± 0.11, 1.20 ± 0.09, 1.05 ± 0.08 folds). After PHx, H1 and H3 groups showed decreased expression of Albumin (0.88 ± 0.05 fold, 0.68 ± 0.03 fold) as compared to their respective control groups (1.00 fold). At day 5, the Albumin expression increased to 1.00 folds and maximum expression was noted at day 7 and day 14 (1.26 ± 0.10 fold and 1.10 ± 0.09) as compared to their respective control groups

(1.00 fold). CK19 expression was significantly higher after 1 day of PHx 11.44 ± 2.45 folds as compared to control groups 1.00. Expression of CK19 increased until 5th day (H3 = 14.29 ± 0.98, H5 = 18.92 ± 1.64). After 5th day of PHx, the expression of CK19 was dropped to 5.4 folds in H7 group. The expression of HNF-4α was up regulated only at day 1 and then down regulated in all other groups. There was 2.99 ± 1.25 fold increase in the expression of HNF-4α in H1 group as compared to control C1 group. Other hepatectomy groups (H3, H5, H7 and H14) showed decreased expression of HNF-4α (0.50 ± 0.06, 0.49 ± 0.44, 0.31 ± 0.32 and 0.50 ± 0.32 folds) compared to their respective control groups (Figure 3A). Values were expressed as mean ± SD. ****P-value ≤ 0.0001 was considered statistically significant vs. control. Fold increase in the expression of Albumin and HNF-4α. Values were expressed as mean ± SD. P-value was not ≤ 0.05.

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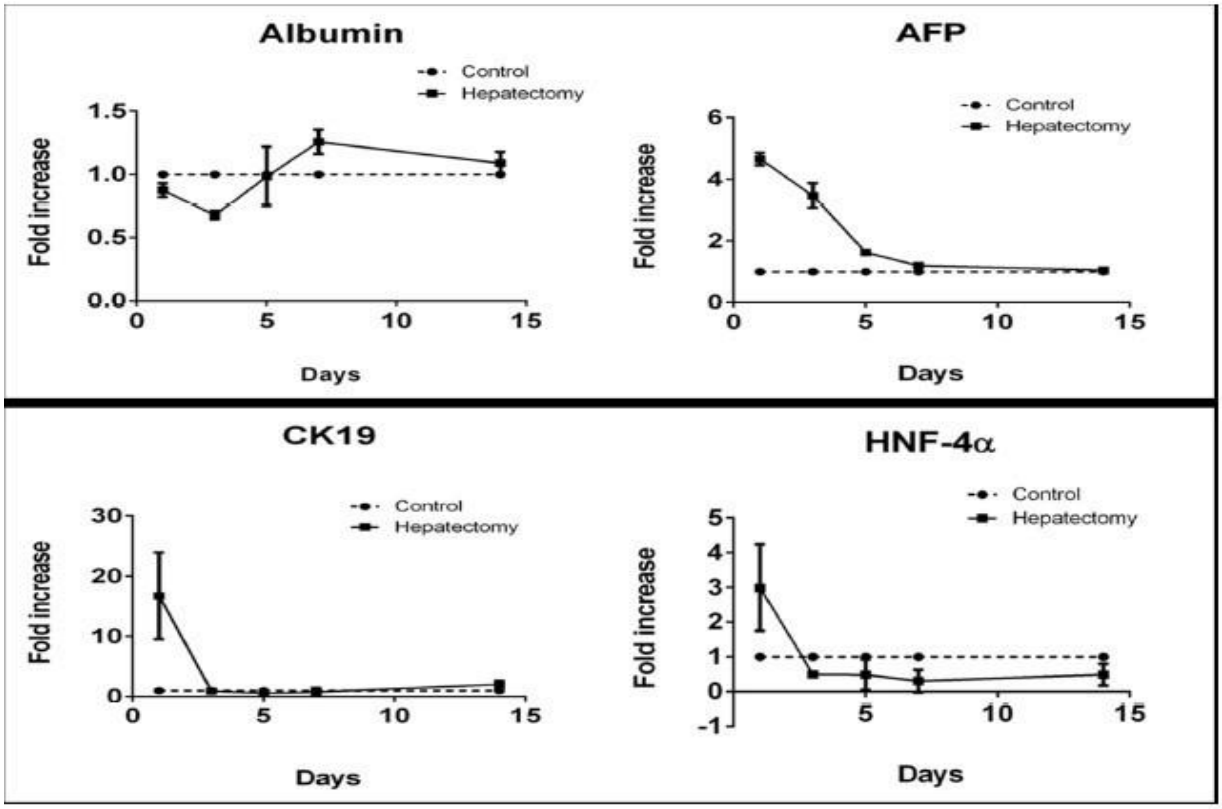

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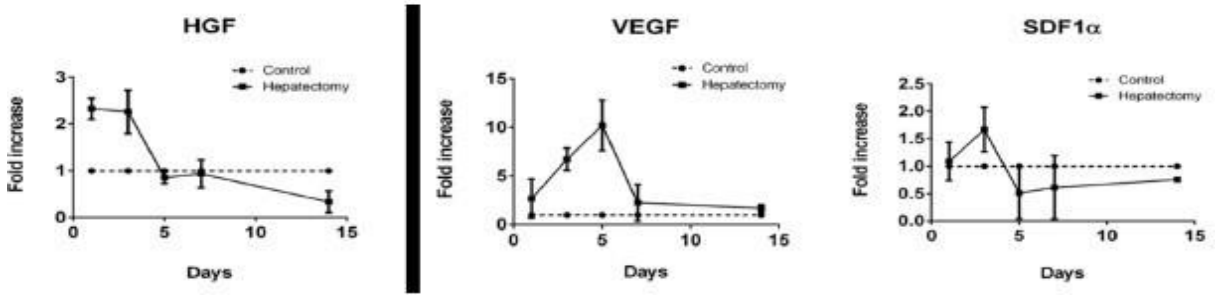

Figure 3-A: Fold increase in the expression of AFP and CK19 **B:** Fold increase in the expression of HGF

HGF expression was remained up-regulated until day 7 and was decreased at day 14. In H1 and H3 groups, there was 2.33 ± 0.23 and 2.26 ± 0.47 folds increase in the expression of HGF as compared to their respective control groups. H5, H7 and H14 groups showed decreased expression of HGF (0.86 ± 0.16, 0.94 ± 0.3 and 0.34 ± 0.23 folds) as compared to their respective control groups. Similarly, VEGF was remained up-regulated until day 5 but, start decreasing at day 7. VEGF expression was significantly higher in: H1 = 2.70 ± 2.20, H3 = 6.73 ± 1.14 and H5 = 10.21 ± 2.60, H7 = 2.27 ± 1.90, H14 = 1.69 ± 0.34 folds as compared to their respective control groups. H5 group showed maximum expression of VEGF. SDF1α expression was increased at day3 after PHx and the down regulated uptil day 14. Expression of SDF1α was higher in H1 and H3 groups (1.10 ± 0.35, 1.67 ± 0.40 folds) as compare to their respective control groups. While, its expression was decreased to 0.52 ± 0.47, 0.62 ± 0.58, 0.76 ± 0.04 folds respectively in H5, H7 and H14 groups as compared to their respective control groups (Figure 3B). Values were expressed as mean ± SD. P-value ≤ 0.05 was considered statistically significant vs. control. Fold increase in the expression of VEGF. Values were expressed as mean ± SD. *** P-value ≤ 0.001 was considered statistically significant vs. control. Fold increase in the expression of SDF1α. Values were expressed as mean ± SD. P-value was not ≤ 0.05.

Immunostaining of hepatic genes:

After PHx, H1 and H3 groups showed decreased expression of Albumin as compared to the control group (C1). After day 5, the Albumin expression was progressively increased and maximum expression was noted at day 14. Similar pattern of expression was observed with CK18 (Figure 4) confirming PCR results. Expression of Albumin in all control groups was similar and uniform therefore, only representative picture from C1 group is shown here. Expression of Albumin was increased as liver regeneration progressed (magnification = 200X). Immunostaining of CK18 after PHx. Expression of CK18 in all control groups was similar and uniform therefore, only representative picture from C1 group is shown here. Expression of CK18 was increased as liver regeneration progressed (magnification = 200X).

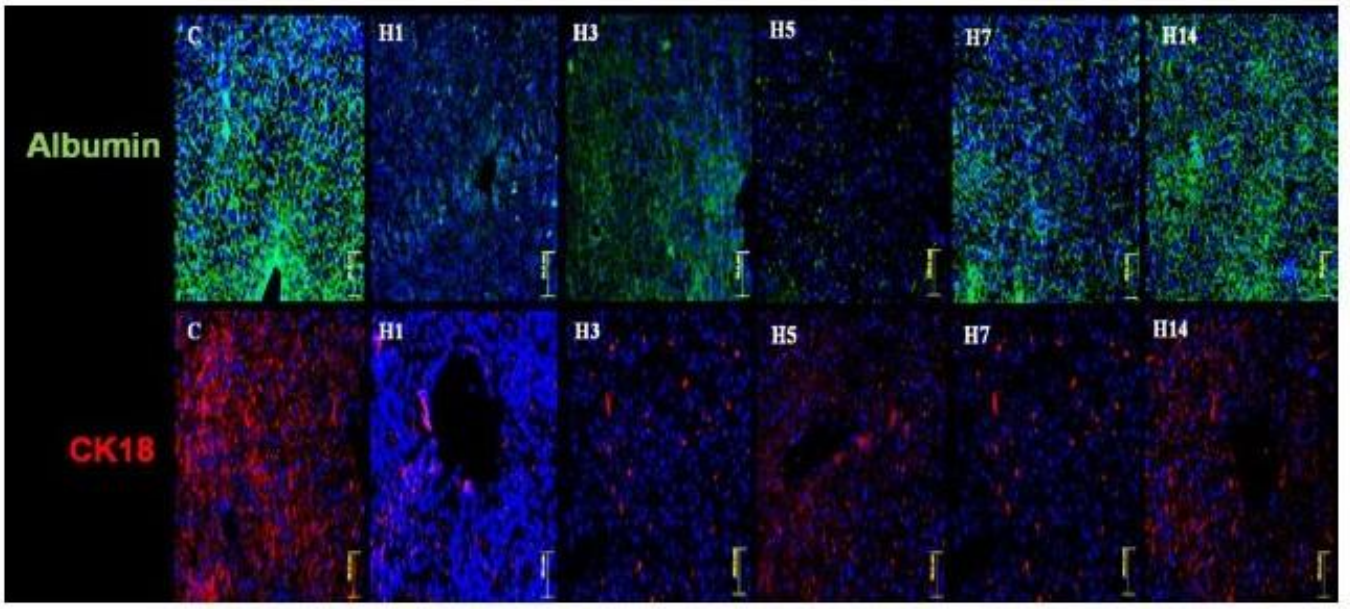

Figure 4: Immunostaining of Albumin after PHx

Estimation of growth factors concentrations after PHx by ELISA:

HGF concentration in serum was increased significantly after hepatectomy and remained high until 7th day of hepatectomy (125.79 ± 0.02 ng/ml in H1, 145.85 ± 0.04 ng/ml in H3, 122.38 ± 0.11 ng/ml in H5, 103.64 ± 0.07 ng/ml in H7 vs. 27.31 ± 0.07 ng/ml in C1, 31.25 ± 0.21 ng/ml in C3, 32.52 ± 0.07 ng/ml in C5, 32.56 } 0.02 ng/ml in C7 groups). In H14 group concentration of HGF was decreased to 46.82 ± 0.01 ng/ml vs. 27.7 ± 0.04 ng/ml in C14 group (Figure 5). ELISA results showed that VEGF concentration was significantly increased in H1, H3 and H5 groups (261.42 ± 2.97 ng/ml, 244.54 ± 0.04 ng/ml and 228.2 ± 1.91 ng/ml respectively) as compared to their respective control groups (112.68 ± 1.35 ng/ml in C1, 121.40 ± 1.15 ng/ml in C3, 114.06 ± 4.34 ng/ml in C5 respectively). On 7th day after PHx, VEGF concentration was decreased to 143.40 ± 2.01 ng/ml in H7 group vs. 121.98 ± 0.11 ng/ml in C7 group and eventually reached to 91.65 ± 2.40 ng/ml in H14 group vs. 97.47 ± 1.24 ng/ml in C14 group (Figure 5). SDF1α concentration in serum of H1 group was slightly lower (5.61 ± 0.12 ng/ml) than C1 group (6.32 ± 0.07 ng/ml). On 3rd day of PHx, concentration of SDF1α was increased to 7.99 ± 0.14 ng/ml in H3 group vs. 6.89 ± 0.06 ng/ml in C3 group followed by a decrease on 5th and 7th day (5.73 ± 0.31 ng/ml in H5 and 5.76 ± 0.35 ng/ml in H7 vs. 6.59 ± 0.04 ng/ml in C5 and 6.35 ± 0.24 ng/ml in C7 groups). SDF1α concentration again increased comparable to normal control on 14th day of hepatectomy (6.50 ± ng/ml in H14 vs. 6.71 ± 0.17 ng/ml in C14) (Figure 5).

The concentration of the Albumin in serum was significantly lowered in H1, H3 and H5 groups (11.57 ± 1.41 ng/ml, 14.55 ± 0.84 ng/ml and 33.98 ± 2.75 ng/ml respectively) as compared to their respective control groups (46.24 ± 0.54 ng/ml, 45.70 ± 0.15 ng/ml, 47.38 ± 0.40 ng/ml). Albumin concentration was increased comparable to the concentration of normal control groups as liver regeneration progressed to 7th and 14th day of PHx (42.27 ± 2.05 ng/ml in H7 and 46.50 ± 1.48 ng/ml in H14 vs. 51.27 ± 0.25 ng/ml in C7 and 51.12 ± 0.33 ng/ml) (Figure 5). Concentration of different proteins was quantified for H1, H3, H5, H7 and H14 groups compared to their respective control groups. Values were expressed as mean ± SD. ****P-value ≤ 0.0001 was considered significant vs. control. Albumin concentration was gradually increased as liver regeneration progressed from H1 to H5 group. HGF levels were almost unVEGF concentration remained significantly higher in H1, H3 and H5 groups compared to their respective control groups. Values were expressed as mean ± SD. ***P-value ≤ 0.001 was considered significant vs. control. SDF1α concentration was higher in H3 group compared to its respective control group. Values were expressed as mean ± SD. P-value was not ≤ 0.05. Values were expressed as mean ± SD. ****P-value ≤ 0.0001 was considered significant vs.
control.

Liver function assays after PHx:

ALAT was observed as the very first biomarker released after partial hepatectomy. On day 1 of hepatectomy, ALAT concentration was 459.8 ± 8.6 IU/L (H1) vs. 40.73 ± 3.25 IU/L in control of same respective group (C1). ALAT concentration decreased gradually to normal control on 7th and 14th day of hepatectomy (234.33 ± 4.5 IU/L in H3, 189.84 ± 56.53 IU/L in H5, 46.61 ± 2.07 IU/L in H7 & 47.64 ± 2.55 IU/L in H14) (Figure 5B). An immediate decrease was observed in ALP on day 1 of hepatectomy (72.16 ± 5.19 IU/L in H1) as compared to control (101.96 ± 4.83 IU/L in C1). There was significant increase in the concentration of ALP at 3rd, 5th, and 7th day of hepatectomy (150.94 ± 5.17 IU/L in H3, 346.61

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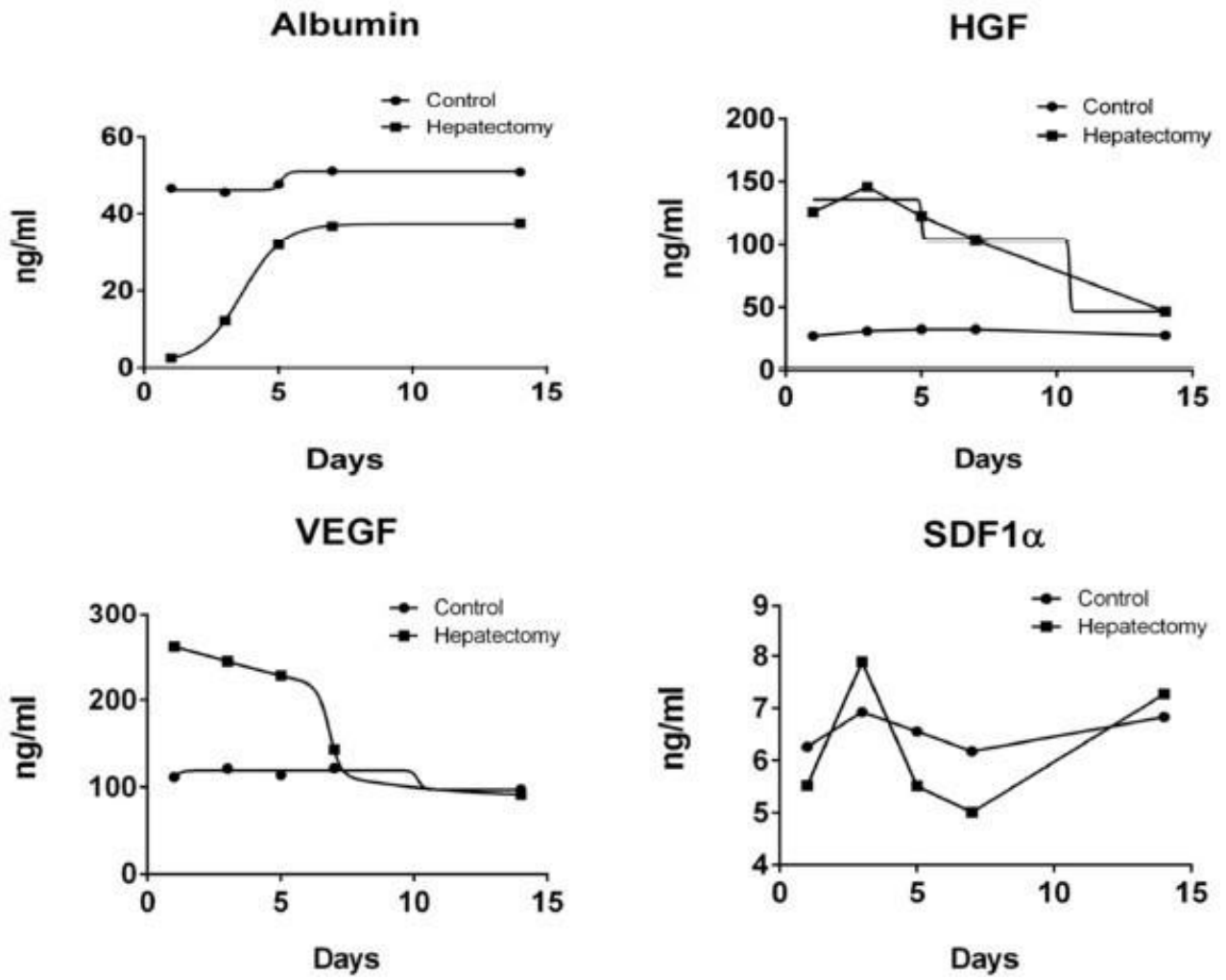

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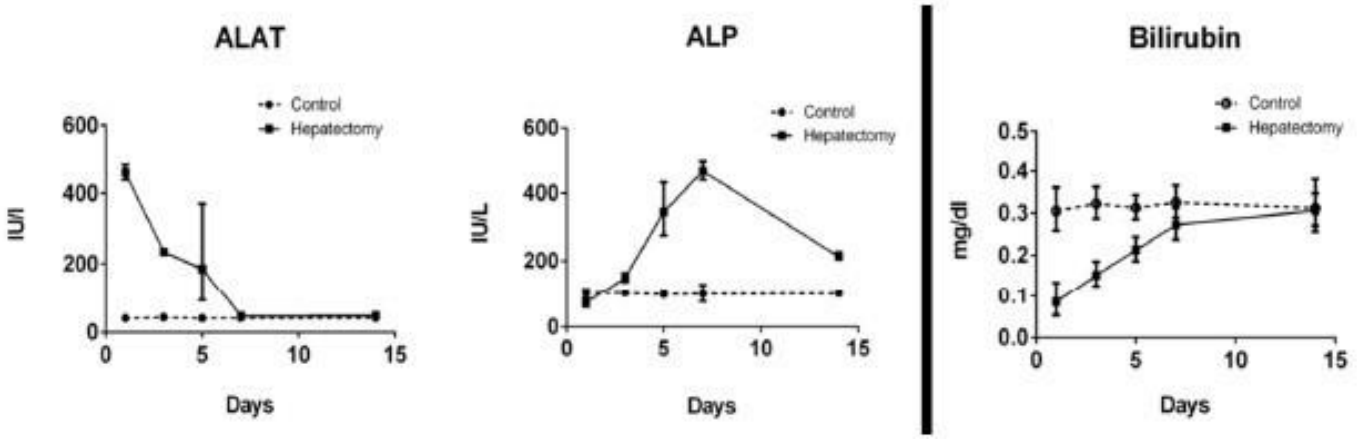

Figure 5-A: ELISA based quantification of albumin, HGF, VEGF and SDF1α concentration after PHx

B: ALAT concentration after PHx

± 32.10 IU/L in H5 and 466.47 ± 10.65 IU/L in H7) as compared to the controls of respective days (101.53 ± 2.43, 97.47 ± 5.15, 99.39 ± 10.26 IU/L). ALP concentration decreased after 7th day of hepatectomy (H7) to 213.5 ± 4.83 IU/L in H14 group (Figure 5B).

Bilirubin was decreased significantly to 0.09 ± 0.02 mg/dl in H1 group as compared to the C1 group (0.31 ± 0.02 mg/dl). The concentration of bilirubin increased gradually as the liver regenerated on 3rd, 5th and 7th day after hepatectomy (0.15 ± 0.01 mg/dl in H3, 0.21 ± 0.01 mg/dl in H5, 0.27 ± 0.01 mg/dl in H7) and eventually reached to the concentration of normal control (0.31 ± 0.02 mg/dl in C14 and H14 groups) on 14th day of hepatectomy (Figure 5B). ALAT concentration remained significantly higher in H1, H3 and H5 groups compared to their respective control groups. Values were expressed as mean ± SD. ****P-value ≤ 0.0001 was considered significant vs. control. ALP concentration after PHx. ALP concentration remained significantly higher in H3, H5, H7 and H14 groups compared to their respective control groups. Values were expressed as mean ± SD. **** P-value ≤ 0.0001 was considered significant vs. control. Bilirubin concentration after PHx. Bilirubin concentration remained significantly lowered in H1, H3 and H5 groups compared to their respective control groups. In H7 and H14 groups, bilirubin was increased to normal control groups. Values were expressed as mean ± SD. ***P-value ≤ 0.001 was considered significant.

Estimation of growth factors by western blot analysis:

Western blot analysis confirmed the findings of PCR and ELISA. HGF expression was increased in H1 and H3 groups and decreased in H5, H7 and H14 groups. VEGF was also remained higher until H5 and then decreased in H7 and H14. Expression of SDF1α was shown minor increase in H3 group but remained lower in other hepatectomy groups. β-actin was used as internal control (Figure 6). Expression of HGF and VEGF was increased at early days of liver regeneration and then decrease was noticed. There is minor increase in the expression of SDF1α in H3 groups while, other hepatectomy groups showed no increase in the expression of SDF1α. B-actin was used as internal control.

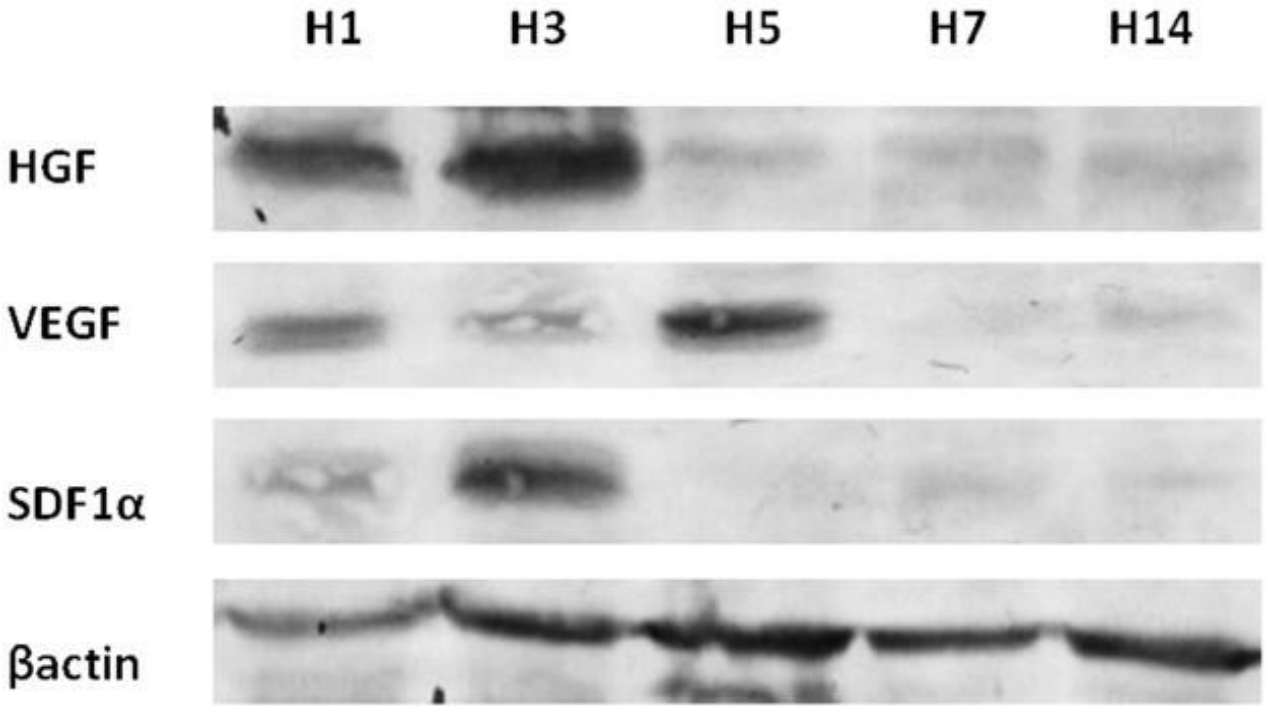

Figure 6: Expression analysis of growth factors after PHx

Discussion:

Liver is an organ with distinctive self-regenerative capacity. A thorough understanding of the mechanisms involved in liver regeneration has gained prime importance because of its emerging impact in different clinical applications such as treating liver diseases, bioengineering, tissue replacement technologies and *in vitro* organogenesis. Gene expression profiling is one of the main aspects which can decipher the process of liver regeneration noticeably and can be further used in regenerative medicine. In the present study mouse partial hepatectomy model was employed to elucidate the molecular circuitry that responds the regeneration phenomena of liver during different stages.

Partial hepatectomy in rodent models is a commonly used technique to study liver regeneration, acute liver failure, hepatic metastasis, hepatic function, and metabolic response to injury [6, 14-16]. Previously, PHx has been extensively studied for shorter time duration with maximum 120 hours [17]. However, this study was planned for the first time to investigate PHx model for a longer period of two weeks. The body weight was used as an aspect to calculate the percentage rate of liver regeneration in accordance with the previous reports [18, 19]. Measurement of the rate of regeneration showed that the liver regeneration is an irregular phenomenon with rapid increase in the beginning approaching a certain level in the middle and then slight decline by the end of the study period. These results are according to the previous studies that validates the kinetics of liver regeneration during early stages was maximum which later directed by petite signal of apoptosis to compensate the regenerated mass of liver [20, 21].

Liver regeneration is known to undergo a series of events such as activation, proliferation, differentiation and survival of mature liver cells directed by several cytokines and growth factors [22, 23]. It has been shown that cytokines play role mainly in the priming of the hepatocytes and growth factors act after cytokines in the proliferation phase [2, 24]. As most of the prior data focuses on the initial phases therefore much systematic data is available for expression of cytokines but not for growth factors [25, 26]. current study however, expression regulation was observed for several physiologically important growth factors i.e. HGF, VEGF, and SDF1α. Several hepatic markers were also investigated to ensure the regeneration process and characterization of hepatocytes. It was observed that AFP level is high in the regenerating tissues of early days as compared to their respective normal controls and decreased after 5 days. AFP is a marker of proliferating liver progenitor cells and proliferating hepatocytes [27]. In mice, CCl4 injury resulted in increased number of hepatocytes expressing AFP between 3 and 4 days of post injury [28, 29], while in current study, the increased expression of AFP elucidates the proliferation of hepatocytes after PHx and correlating well with the proliferating cells in both cases. Albumin is the mainly prolific protein present in the differentiated liver cells symbolized as hepatocyte marker [30], while Cytokeratin19 (CK19) is a biliary cell marker [31]. In present study, expression of both Albumin and CK19 enhanced with the proliferation and differentiation of hepatocytes in a time dependent manner. Cytokeration18 (CK18) is another hepatocyte marker used in the study. Its expression remains [32], while on the other hand CK18 also known as a biomarker of liver injury and hepatocyte apoptosis. It is a major intermediate filament protein consisting about 5% of total protein in liver and a famous substrate of an apoptotic marker caspase during hepatocyte apoptosis. The number of cells eradicated by apoptosis is equivalent to the number of cells produced by mitosis in a healthy liver showing the proper organ homeostasis [33, 34].

Expression analysis of one of the liver enriched transcription factor, Hepatocyte nuclear factor-4 alpha (HNF-4α) in current study revealed its enhanced expression only during first 24 hour, declined and remained steady in the remaining duration. Previous studies reported that HNF-4 α, a zinc finger protein, binds to the hepatocyte specific DNA regulatory region initiating the transcriptional assembly [8, 35-37]. It is involved in the regulation of more than 1000 genes that directly correlates with the hepatocytes proliferation and functioning [38-41]. Hence it demonstrates that it is a marker of not only regeneration but enhanced the potential of other proliferation markers.

Gene expression analysis through real time quantitative PCR, western blot and ELISA indicated an enhanced level of hepatocyte growth factor (HGF) during first 3 days after hepatectomy leading to a significantly gradual decrease till day 14. In contrast, control mice did not show any significant change in the expression of HGF throughout the study period of 14 days. Increased levels of HGF till 3rd day post PHx depict its crucial role in initiation phase as it has been previously described as an initiator of liver regeneration due to its direct mitogenic effect on hepatocytes [9, 42, 43]. Results in the study are in accordance with the reported literature as HGF induces the DNA synthesis in hepatocytes when checked in vitro in a serum free media and cause liver enlargement when injected in vivo [10, 44, 45]. HGF operates in a biphasic manner after PHx as hepatocytes go through two or three rounds of replication. The first peak of DNA fabrication noticed approximately after 36 hours in mouse and then undergoes in second phase of hepatocytes DNA synthesis [5, 46]. Moreover, HGF mRNA transcription is known to be stimulated by circulating norepinephrine [47], or insulin-like growth factor [48].

Vascular endothelial growth factor (VEGF) is a central and significant growth factor involved in both angiogenesis and vasculogenesis [49]. In the present study, it has been demonstrated that expression of VEGF after PHx constantly increased till day 5 and then a sharp decrease was noticed on day 7 till day 14 reaching the value of normal control. Increased level in early days after PHx showed that VEGF plays a definitive role in the proliferation of hepatocytes and not in later stages as it has been previously documented by Taniguchi et al., 2001 [50]. They illustrated that the level of VEGF reach to a maximum level after 72-96 hours. It has been also revealed that augmented expression of VEGF mRNA in both hepatocytes and non-parenchymal cells showed immense importance of VEGF in liver regeneration [51, 52]. VEGF initiated the regeneration process through activating the sinusoidal endothelial cells after 24 hours of PHx and then reached towards the maximum expression level by activating the proliferation of hepatocytes until second phase of their division [12, 53].

Stromal cell-derived factor 1α (SDF1α), another important growth factor known to be involved in liver regeneration was also studied in PHx model. Both qualitative and quantitative analysis showed that the expression of SDF1α was minor in both PHx and control mice throughout the study period. SDF1α secreted during tissue damage, directs tissue-committed stem cells essential for organ/tissue regeneration. CXCR4 is a specific receptor present on the liver progenitor cells and SDF1α is known to activate these cells at the site of injury via its specific binding with CXCR4 [13, 54, 55]. Another study showed that SDF1α released at the site of injury and recruited CXCR4-positive stem cells to the site of injury [56, 57]. Contrary to the severe hepatic injury where SDF1α mediated oval cell repair is involved, moderate hepatic injury involves the proliferation of hepatocytes. Therefore, minor expression of SDF1α in the present study determined that the regeneration of liver involved the proliferation of hepatocytes and rule out the role of hepatic progenitor cells.

Functional improvement in liver after PHx is essential to counter postoperative liver deficiency. Accordingly, in the present study,

different markers of liver function were assessed based on their sequential changes occurred during liver regeneration. Interestingly, a marked increase was observed in ALAT release during early days of regeneration that directed towards baseline on day 7. Previous studies showed that ALAT is released during necrosis of hepatocytes [58, 59], hence its level increased in the initiation leading towards normal level as the liver regenerates. Another sensitive indicator of liver function, ALP was investigated during the study period depicting a marked increase in ALP level till day 7, showed the proliferation of hepatocytes and reached near the level of control on day 14th. In previous studies, this marker has been exemplified for hepatocyte proliferation and integrity and change in its level showed the obstruction of hepatobiliary system [60-62]. Cancerous hepatocytes also produce large amount of ALP as these cells proliferate very rapidly [63]. Bilirubin level in this study also supports the liver functionality post PHx as its level amplified with progression in liver regeneration meeting the normal titer. It is demonstrated in the previous studies that bilirubin level improved concurrently with liver volume recovery [64, 65]. Therefore, bilirubin level confirmed the functional improvement in liver.

Conclusions:

It is concluded from the data that the expression regulation of different growth factors is crucial in the liver regeneration after PHx. Results further demonstrated that hepatocyte proliferation is mainly involved in the recovery of liver whereas, expression of HGF and VEGF has direct correlation with hepatocyte proliferation. Profiling of growth factors can not only be used for the in vitro differentiation of hepatocytes but also improving their survival for the development of hepatocyte reservoir. Therefore, current study provides a very helpful paradigm for the *in vitro* organogenesis of liver and implication of this profile to treat human liver diseases.

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