Introduction
Liver cirrhosis is the 11th leading cause of death worldwide, accounting for 1 billion deaths annually.[1] Hepatitis C and B infection, non-alcoholic fatty liver diseases, non-alcoholic steatohepatitis, and alcohol abuse are the main factors that lead to liver injury and ultimately hepatic cirrhosis in humans.[2] Hepatic fibrosis, the precursor to cirrhosis, leads to excessive accumulation of extra cellular matrix proteins in the liver due to chronic injury.[3] When the injured tissue is replaced by a collagenous scar during fibrogenesis, cirrhosis presents itself as impaired liver function and portal hypertension.[4] Development of major clinical complications of cirrhosis, such as ascites, hepatic encephalopathy, renal failure, and variceal bleeding, is associated with reduced survival rates, and liver transplantation is the only treatment available to
such patients. Therefore, alternative options, such as regenerative therapy, need to be explored to compensate for the shortage of donor organs and high mortality rate. In this study, we investigated the regenerative efficacy of adipose-derived stromal vascular fraction (SVF) in combination with autologous activated platelet rich plasma (aPRP) on Carbon Tetrachloride (CCL₄)-induced liver fibrosis in rats.

Data from clinical trials shows that SVF has been used to treat diseases such as orthopedic disorders, hepatic failure, inflammatory diseases, and autoimmune diseases. Immuno-modulatory, anti-ulcer, and anti-inflammatory properties are the hallmark of SVF along with the capacity to secrete bioactive proteins such as cytokines, chemokines, and growth factors. It is also a rich source of self-renewing and multi-potent mesenchymal stem cells (MSCs), making it an excellent regenerative tool that has been a topic of extensive research over the last few years. Intravenous transplantation of adipose-derived stem cells (ADSC) in acute liver failure rat models has shown restoration of liver function and increase in survival rates. A study conducted on CCl₄-injured rat liver models has shown that human chorionic plate-derived mesenchymal stem cells (CP-MSCs) isolated from the placenta demonstrated promising therapeutic effect on the liver as indicated by reduced blood levels of AST, ALT, and total bilirubin along with decreased deposition of type I collagen. Moreover, immunosuppressive properties of ADSCs allow them to be transplanted into a recipient irrespective of human leukocyte antigen (HLA) match with donor. Several recent studies demonstrated that the use of autologous PRP along with ADSCs enhances tissue regeneration. PRP has beneficial effects on CCl₄-induced rat liver fibrosis as it releases growth factors that promote recruitment, adhesion, and proliferation of stem cells. Some preclinical studies also ascertain that PRP acts as a scaffold to increase the potential of transplanted stem cells in wound healing, bone regeneration, and tendon repair.

Since CCl₄-induced rodent models have been extensively relied upon to study liver injury and regeneration, we also employed CCl₄-induced SD rats as a model of liver fibrosis and injected them intravenously with SVF and autologous PRP. Evaluation of various parameters during the treatment showed that SVF and autologous PRP have a synergistic regenerative outcome on rat liver fibrosis. The findings can open doors to effective stem cell-based treatment of liver fibrosis in humans where routine therapeutic methods fail and long transplant waiting lists lead to death.

Materials and Methods

Ethics Statement and Animals
Thirty male Sprague Dawley rats of age 8 to 10 weeks, weighing between 160 and 200g, were obtained from the Animal House Facility of the Preclinical Safety Assessment Division, Wockhardt Research Centre, Aurangabad, India. The animals were housed individually in standard stainless-steel cages in a controlled environment, with temperature and humidity maintained at 25 ± 3°C and 30 to 70%, respectively, and 12-hours light/dark cycles. They were handled humanely throughout the experiment and had free access to standard pelleted laboratory animal diet and RO treated water. Each cage was identified with a unique label having details like project number, cage and animal number, study title, species/strain, animals/group, individual animal identification, and important dates. Animals were marked for identification with 5% picric acid.

The study was initiated only after the protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Wockhardt Research Centre, Aurangabad, India (Approval No. B/14/ 2013-14). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Isolation of Stromal Vascular Fraction (SVF)
SVF was isolated from adipose tissue of SD rats. Briefly, about 3 to 6g of fat was collected, washed, finely minced, and allowed to digest in collagenase type II (Thermo Fisher Scientific, Waltham, MA, USA) (1% in 1XPBS) at 37°C for one hour with intermittent shaking. The digested fat was then centrifuged at 300g for 10 mins. The precipitated cell pellet was aspirated gently from the bottom of the tube and washed thrice with 1X PBS. The resulting SVF was divided into three parts. Each part was used for the three time-points of treatment. The cells were stored in liquid nitrogen in the time period between doses.

Preparation of Activated Platelet Rich Plasma (aPRP)
aPRP was obtained from blood of SD rats. Approximately 1ml blood was collected under light anesthesia from seven animals by retro-orbital bleeding into tubes containing anticoagulant. The pooled blood (7 mL) was centrifuged at 900 rpm for 15 mins. The separated straw-colored plasma was collected in a sterile tube and centrifuged at 2800 rpm for 10 mins. A button of platelets was obtained as the pellet. This pellet was re-suspended in 900 µl of the supernatant plasma to obtain platelet rich plasma (PRP) and the remaining supernatant was discarded. Calcium chloride (175 µl) was added to the PRP and kept at 37°C for about 30-40 mins till a clot was formed. The clear fluid surrounding the clot containing growth factors released from the platelets was collected in a fresh tube and labelled as activated Platelet Rich Plasma (aPRP). The aPRP was divided into aliquots and stored at -20°C for subsequent use.

Induction and Maintenance of Fibrosis
Fibrosis of the liver was induced in SD rats by oral administration of Carbon tetrachloride (Purity ≥99%,
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Merck KGaA, Darmstadt, Germany) diluted in Corn oil (Across Organics, Fair Lawn, New Jersey, United States) at the proportion of 1:1. Diluted CCl₄ was administered orally at a dose rate of 200 µL per animal twice a week initially and on the basis of severity of ALT/AST ratio, effect on body weight gain and mortalities, dose was re-adjusted between 100–150 µL per animal. Dosing of CCl₄ was continued for 12 weeks. Liver damage was monitored by periodical biochemical assessment of the enzymes AST and ALT. At the end of the 12-week period, mortality was observed in 14 animals due to severe liver damage; 16 out of 30 animals were allotted for the experiment.

**Experimental Design**

Experimental outline is explained in Fig. 1. The 16 animals with elevated ALT and AST were randomly allocated to the control and treatment groups, with eight animals in each group. Group I – Control, Animal ID one to eight and Group II – Treatment, Animal ID nine to sixteen. The treatment formulation consisted of minimally manipulated adipose derived SVF combined with aPRP. The SVF cell density in the formulation was two million cells per dose. The SVF-aPRP was administered intravenously via lateral tail vein in the treatment group animals. The control group animals received normal saline at the same dose volume. Three consecutive doses were given at one-week interval. After the last dose, animals were observed for 14 days and sacrificed on day 35 post treatment initiation.

**Parameters Evaluated**

**Body Weight:** Body weight of individual rat was recorded on the day of randomization and at least once weekly prior to the commencement of treatment and during treatment.

**Clinical Signs:** All animals were observed twice daily for any signs of illness or behavioral changes. All clinical signs were recorded in terms of time of onset, duration, and severity.

**Mortality:** The animals were observed twice daily; once in the morning and once in the evening for morbidity and mortality.

**Biochemical Parameters:** Levels of liver enzymes AST and ALT were analyzed as indicators of hepatotoxicity. The enzyme levels were monitored periodically during the Fibrosis induction period and recorded at the end of the induction period (before start of SVF-aPRP treatment). The levels were also recorded on Day 7, Day 14, and Day 28 of the study. Blood samples of individual rats from both groups were withdrawn from the orbital sinus and collected in tubes without anticoagulant for analysis. The ALT and AST in serum were estimated by using automatic biochemical analyzer, Dimension Xpand, manufactured by Dade Behring, USA using standard reagent kits (Dade Behring, USA).

**Pathological Examination:** Animals from both the groups were sacrificed after completion of 14 days from the last dose of SVF- aPRP treatment and subjected to complete gross pathological examination including external and visceral examination. Gross lesions of liver were graded as per the appearance of the parenchyma. Liver tissues were preserved in 10% neutral buffered formalin for histo-pathological evaluation.

**Histopathological Examination:** The liver tissues from all the animals were fixed and subjected to paraffin embedding followed by sectioning and haematoxylin and eosin (H&E) staining. Histopathological liver changes were scored as per method stated by Lee et al.

Grade 0: No abnormality detected, Grade 1: Short collagenous septa extend from central veins,

Grade 2: Slender septa link the central veins but lobular architecture is preserved,

Grade 3: Pseudolobuli form thin septa,

Grade 4: Parenchyma is subdivided into pseudolobuli by thin septa

**Statistical Analysis:** All statistical analysis and graph generation were performed using GraphPad Prism version 9.1.1. 2-way ANOVA was used to analyze the AST ALT data. p < 0.05 was considered statistically significant.

**RESULTS**

**Liver Enzymes AST and ALT**

At the start of the study, 30 animals were administered with CCl₄ for a span of 12 weeks to induce liver fibrosis. 14 animals died during the induction period of liver fibrosis. The 16 animals which survived, were randomly divided into 2 groups – treatment and control. The ALT (mean: 1772 Units/L, SD: 844) and AST (mean: 710 Units/L, SD: 572). Three doses of SVF and aPRP were prepared and dosed at weekly intervals. 1st dose was administered on day 0, 2nd dose on day 7 and 3rd dose on day 14. By day 7 and Day 14 reduction of ALT and AST was seen, (Figs. 2 and 3) in both control and treatment groups, without any significant difference compared between the 2 groups. On Day 14, it was observed that both the ALT and AST levels increased in both groups with no statistical significance. On day 28 the ALT levels in the untreated control group increased from 104 to 123 Units/L (Fig. 2), however, the treatment group showed no increase. Similarly, the AST levels (Fig. 3) observed between day 14 and day 28 showed
a significant increase in the untreated control group (240 to 357 Units/L) as compared to the treatment group (171 to 216 Units/L).

On Day 28 the animals were terminally sacrificed, both the ALT and AST levels on day 28 we found a statistically significant difference between the control and treated group. To corroborate this, difference in gross morphology, showed difference between two groups. Histopathological findings also showed difference in fibrosis, bile duct and hyperplasia. Results indicate, treatment with SVF and aPRP may have regenerative effects.

Gross Morphology of Liver
Grading of liver from control and treatment group was done based on gross morphological evaluation. On day 28 of the study, animals from both groups were sacrificed and livers removed for gross pathological examination. As cirrhosis is characterized by nodule formation and scarring of the liver tissue, the lesions in liver were graded as per the appearance of the parenchyma (Table 1, Fig. 4).

At the end of treatment, 50% of the animals from control group showed scarring of the liver parenchyma (Grade 3), however, only 12.5% animals from the treatment group showed the similar findings (Table 1).

A normal, non-cirrhotic liver has smooth and glossy surface without scars or depressions (Fig. 5-A). The liver of all the animals in the control and treatment groups (Fig. 5B-G), showed uneven surface and depressions over the lobes, which were identified as fibrosis histologically. The extent of fibrosis appeared to be less marked in the treatment group. Most of the animals from the SVF treatment group showed mild depressions and uneven surfaces and only 12.5% showing liver scarring. However, 50% of animals from control group showed marked depressions and tissue scarring; suggesting the positive therapeutic effect on liver Fibrosis in SVF–aPRP treated animals.

Histopathological Evaluation
Histopathological evaluation of the liver tissue was performed by H&E staining. Histopathological liver damage was scored as per method stated by Lee et al.\textsuperscript{21}

The tissue of normal non-cirrhotic liver showed regular cellular structure (Fig. 6-A). In the control group (Figs 6-B, 6-C, 6-D), most animals showed high grade of fibrosis with a distorted cellular architecture due to pseudolobuli formation by thin septa. The treatment group showed much lesser extent of fibrosis as slender septa were observed but the parenchymal architecture was preserved (Figs 6-E, 6-F, 6-G). It is pertinent note that the severity of fibrosis was markedly reduced in SVF-apRP treated animals compared to untreated animals suggesting that SVF-apRP treatment in cirrhotic liver would be beneficial with little modification of dose and duration (Fig. 7)

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Table 1: Grading of gross morphological changes in liver, gross morphology grading of liver on day 28 post initiation of treatment...
Reversal of CCl₄ induced liver fibrosis by Adipose SVF and activated PRP.

Discussion

Chronic liver diseases constitute a global challenge. Current medical treatments for these diseases have achieved limited efficacy. All chronic liver diseases can lead to hepatic fibrosis and eventually liver failure. There is an urgent need to develop treatment modalities to address this unmet medical need, which left untreated eventually has a fatal outcome. It is in high demand to find new medicines for the treatment of liver diseases and control of fibrogenesis. Regenerative approaches for addressing this have been reported and our study has attempted to gain an insight into the synergistic effect of SVF and aPRP as a regenerative therapy for the treatment of liver fibrosis and impaired liver function.

CCl₄-induced liver damage in rodents has been widely used as an experimental model.[15,18,19] During the induction period, mortality was observed in 14 animals, which is comparable to mortality rates of 30 to 60% observed in other studies.[22-25]

Assessment of residual hepatic functional reserve is indispensable for stratifying the severity of liver fibrosis and fibrosis. No single marker is entirely reliable for predicting residual function since hepatocytes possess a wide array of different functions. Tests of this type provide more information than simple hepatic histologic features about the progression of liver fibrosis, irrespective of the aetiology. We measured the serum AST and ALT levels to determine the degree of residual hepatic functional reserve in the rats (Figs. 2 and 3). Significantly lower

Fig. 5: Gross appearance of liver from untreated and SVF-aPRP treated animals. Images showing gross macroscopic appearances of livers from different experimental groups. A - normal non-cirrhotic liver from a healthy animal; B, C, D - livers of animals from the control group showing uneven surfaces with markedly depressed structures; E, F, G - , India

Fig. 6: Images showing histopathology staining (10X) of livers from different experimental groups. A - normal, non-cirrhotic liver from a healthy animal showing regular cellular architecture; B, C, D - livers of animals from the control group showing high grade of fibrosis characterized by parenchyma that was subdivided into pseudolobuli by thin septa; E, F, G - livers of animals from the treatment group showing lesser extent of fibrosis.

Fig. 7: The scoring of gross pathologies of liver, a comparison of treated vs untreated. Notable changes were observed in the grades of fibrosis and cirrhosis between the treated and untreated, indicating that the SFV-aPRP combination had a beneficial effect on treated animals.

levels of the serum AST and ALT were resulted from the treatment group compared to the control group.

ALT is a more specific marker of liver damage than AST.[20,26] At the end of the fibrosis induction period, the mean AST and ALT values of the 16 surviving animals were 612 IU/L and 1350 IU/L, respectively, indicating that significant liver damage had been induced.

Macroscopic evaluation of the liver tissue showed improved reversal of liver injury in the treatment group over the control group (Table 1). Gross pathological observation showed scarring only in 12.5% of the treatment group animals, as against 50% in the control group. The histo-pathological analysis showed significantly reduced tissue damage in the SVF-aPRP treated animals, with lesser extent of fibrosis (Fig. 5). The appearance of the parenchyma observed post 28 days of treatment revealed a significant difference between the treated and untreated groups, suggesting that the SVF+aPRP had a possible regenerative effect on the liver. Various etiologies induce chronic inflammation, and the normal lobular architecture of the parenchyma is replaced by micro-nodules characterized by pseudolobuli formation. The macroscopic presentation of livers of treatment group suggested a reversal in treatment group based on the fibrosis, fatty change, bile duct hyperplasia and cirrhosis grade scores. This could be attributed to the anti-inflammatory effect of the aPRP and SVF, as has been reported in literature.

Upon reviewing the data, we realized the study has several limitations that need to be addressed to confirm the regenerative potential of SVF and aPRP in this model. Notably, the profile of liver enzymes should also include the total bilirubin, Albumin, ALP (Alkaline Phosphatase), GGT (Gamma-glutamyl transferase), LD (Lactate dehydrogenase) and PT (pro thrombin time). We noticed that the AST and ALT levels showed a significant difference between the 2 groups on day 28, it would be interesting to have followed up the animals for a further period of 2 months to estimate the difference in the biochemical parameters and also the histological changes. To conclusively establish that the combination of SVF and aPRP has a regenerative potential, we also suggest that groups including only SVF and aPRP should have been compared. It would also be interesting to evaluate if there is a dose dependent effect and therefore different doses of cells would help assess the dose for further studies in humans. To better assess the extent of fibrosis special stains such as Mason Trichrome and Picrosirus Red should be used to evaluate collagen deposition.

This study shows the possible role of SVF and aPRP in supporting liver regeneration and function in a rat model of liver fibrosis. Several limitations to the existing study have been identified, and further studies addressing these limitations may lead to a possible alternative future therapy for patients with acute and chronic liver disease.

This therapy could possibly address the current therapy limitations such as the lack of organs for transplantation. However, to enable this research to be used as a clinical treatment, further research is required. Based on this preliminary study, which has demonstrated the SVF+aPRP has a beneficial effect and notable reversal changes were observed both macroscopically and microscopically. We hope that this study lays a foundation for future research into this area of regenerative medicine. Understanding of the mechanism of anti-inflammation, and the role of growth factors and cytokines would be essential in taking this to a treatment level stage, where clinical trials would be required to develop this as a treatment for liver fibrosis.

REFERENCES
Reversal of CCl\textsubscript{4} induced liver fibrosis by Adipose SVF and activated PRP.


