

Three Cases of Alcohol-Induced Acute-On-Chronic Liver Failure With Successful Support by Adipose-Derived Stem Cells

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OBJECTIVES: Acute liver failure (ALF) and acute-on-chronic liver failure (AOCLF) are critical medical conditions with urgent therapy requirements. When ALF or AOCLF are due to alcohol intoxication or based on chronic alcohol abuse, virtually, no therapeutic options are available as liver transplantation is prohibited. In this case series, treatment of alcohol-induced ALF/AOCLF with adipose-derived stem cells (ASC) was tested under compassionate use.

METHODS: ASC from 2 donors were isolated, cultured, and expanded by established protocols. ASC were administered to 3 individuals with either ALF or AOCLF due to alcohol abuse under compassionate use. Clinical presentation, serum measurements, and other diagnostic methods were compiled before ASC treatment and during the disease course after ASC administration.

RESULTS: Three patients were admitted to the Department of Gastroenterology, Hepatology, and Infectious Diseases (University Hospital Magdeburg) with acute or AOCLF due to alcohol abuse. All 3 patients presented in impaired general condition and with elevated, in 1 case drastically elevated, serum liver enzyme concentrations. Treatment with ASC led to improvements in general condition and reduction of serum transaminases. In 2 cases, reduction of liver stiffness and increase of liver function by the C¹³ methacetin breath test were observed after ASC treatment. Recovery to a normal condition was achieved between 1 and 2 months after ASC treatment. No adverse effects associated to ASC treatment were observed.

DISCUSSION: ASC treatment may be a feasible option to enhance recovery from alcohol-induced ALF or AOCLF. ASC treatment seems safe in the presented cases.

Clinical and Translational Gastroenterology 2019;10:e00095. <https://doi.org/10.14309/ctg.0000000000000095>

INTRODUCTION

Liver failure (LF) is a life-threatening clinical syndrome with a variety of causes and high mortality. Depending on the etiology, treatment may be limited to liver transplantation (LT) (1). Problems of LT are organ shortage, immunosuppression-related complications, and exclusion of patients with active alcohol or/and drug abuse (2). Especially for alcohol- or drug-induced LF, new therapeutic approaches are required, as clinical management is still challenging with limited treatment options (3).

In vitro and *in vivo* studies have shown promising results of mesenchymal stem cells treatment for LF (4) and of adipose-derived stem cells (ASC) for regenerative medicine. ASC can be obtained easily from adipose tissue and lipoaspirate (5) and

differentiate into various cell types, including hepatocytes (5,6). ASC are able to secrete hepatocyte protective and promoting factors (7,8). ASC can be applied in autologous, allogenic, and xenogeneic settings due to absent human leukocyte antigen (HLA) expression, without HLA-matching for allogenic ASC treatments (9,10). These properties of ASC may contribute to treatment success for LF in preclinical and clinical studies (4,11); however, exact mechanisms remain unclear (11).

In this report, 3 patients with acute or acute-on-chronic LF (ALF/AOCLF) due to alcohol abuse or acute alcohol toxicity are presented. These patients were successfully treated with ASC under investigative compassionate use. Since the used ASC have not been approved by any authority for this specific treatment and

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Received August 19, 2019; accepted August 29, 2019; published online November 26, 2019

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application was in a mere experimental setting, the main aim of this case series was on safety of ASC treatment in alcohol-induced LF.

MATERIALS AND METHODS

Isolation and expansion of adipose-derived stem cells

Isolation of human allogenic ASC from the stromal vascular fraction was performed according to Zuk et al. (5) and Zhu et al. (12) with modifications to achieve good manufacturing practice (GMP) compliance (13). Briefly, lipoaspirate from 2 healthy female voluntary donors (donor A: 21 years, body mass index = 25.1/donor B: 40 years body mass index = 21.9) was collected. Both subjects gave written informed consent in accordance with the Declaration of Helsinki. The lipoaspirate was washed and digested with Collagenase NB 6 GMP Grade (Nordmark Biochemicals, Uetersen, Germany) according to the manufacturer's recommendations for ~35 minutes at 37 °C. After centrifugation for 10 minutes (400g, room temperature), the supernatant was discarded. For erythrocyte depletion, the cells were further separated by Ficoll centrifugation (400g, room temperature, 30 min [GE Healthcare Bio-Sciences, Pittsburgh, PA]). Afterward, the cells were seeded in a cell culture flask with Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 with 2% KnockOut SR XenoFree Medium (ThermoFisher Scientific, Waltham, MA) and cultivated for 24 hours (37 °C, 6% CO₂, 95% relative humidity). On the next day, cells were washed with phosphate buffered saline (PBS) (without Ca²⁺ or Mg²⁺ [Biochrom, Berlin, Germany]) and expanded with culture media containing 10% (v/v) pooled human serum (Zentrum für Klinische Transfusionsmedizin, Tübingen) and 1% penicillin/streptomycin (Biochrom) for 6–7 days. Afterward, the cells were washed with PBS and cultivated in culture media without antibiotics, till confluency and criteria for ASC according to Bourin et al. (14) were reached (8–14 days, corresponding to passage 0).

Preparation of adipose-derived stem cells for treatment application

Expanded human ASC (passages 0–5) were used for compassionate use in an allogenic setting. To this end, the ASC were harvested with trypsin and washed several times with PBS (without Ca²⁺ or Mg²⁺), suspended in a liquid formulation described in Patent PCT/EP2019/053585 (unpublished), filled in sterile vessels, and stored at 2–8 °C till use at the Department of Gastroenterology, Hepatology, and Infectious Diseases (Otto-von-Guericke University Magdeburg). The isolation, expansion, and preparation of the ASC were performed at Oxacell AG (Potsdam) in accordance with Art. 40 of Directive 2001/83/EC and Art. 13 of Directive 2001/20/EC (Certificate Nr.: DE_BB_01_GMP_2017_1018).

Flow cytometric measurements

To assess the cell number (nucleated cells) and viability, cells were diluted in Guava ViaCount Reagent (Merck Millipore, Darmstadt, Germany) and counted in a Flow cytometer (Guava easyCyte 6-2 L, Merck Millipore) according to the instruction manual.

Afterward, the phenotype of the herein used ASC was verified by surface marker expression. All ASC used for treatment were tested positive for the surface markers CD73, CD90, and CD105 and tested negative for the surface markers CD34, CD45, and HLA-II as recommended previously (14). Briefly, a total of 1.5×10^4 cells were suspended in 100- μ L PBS without Mg²⁺ Ca²⁺ (Biochrom)/2% KnockOut (ThermoFisher

Scientific) and incubated with antibodies coupled to fluorescein isothiocyanate, phycoerythrin, or allophycocyanin. The following antibodies were used: anti-CD34, anti-CD45, anti-CD73, anti-CD90, anti-CD105-FITC, and anti-HLA-DR, DP, DQ (all Miltenyi Biotec). The samples were incubated for 15 minutes in the dark at 2–8 °C and washed with PBS without Mg²⁺ Ca²⁺ (Biochrom)/2% KnockOut (ThermoFisher Scientific) afterward. The samples were suspended in 150- μ L PBS without Mg²⁺ Ca²⁺ (Biochrom)/2% KnockOut (ThermoFisher). Before every measurement, integrity of all channels was verified with an easyCheck-Kit (Merck Millipore). At least 5,000 events were analyzed and compared with appropriate isotype controls. All measurements were performed with fixed gates and protocols according to the GMP guideline. The final results were calculated by subtracting the background (isotype controls).

RESULTS

Cell viability and surface antigen expression

Cell viability and surface marker expression of ASC used for treatment were tested by flow cytometry (see above). All cell passages fulfilled the viability and immune phenotype criteria described by Bourin et al. (14). The results are shown in Table 1.

Case reports

Three patients presenting with acute alcohol toxic LF to the Department for Gastroenterology, Hepatology, and Infectious Diseases (OVGU), who were fitting candidates for investigational compassionate use of ASC and agreed to the procedure. Every patient received 2–4 cell doses of $2.0\text{--}7.8 \times 10^7$ ASCs as intravenous infusion mixed in physiologic salt solution until a significant improvement of the symptoms was recognized. Case I was a female patient with known chronic alcohol abuse and alcohol-induced liver cirrhosis, who was referred from a secondary hospital with suspected AOCLF due to relapse of alcohol abuse. Case II was a female patient with alcohol abuse since 4 years, who was referred from a secondary care hospital with suspected alcohol-induced LF. No overt signs of liver cirrhosis were observed in case II. Case III was a male patient, who was referred from a specialist practice for suspected AOCLF due to alcohol. Basic information on these 3 cases and their condition at admission is given in Table 2. All patients were admitted with some degree of jaundice and impaired general condition. None of the patients exhibited reasonable improvement of their clinical situation under alcohol withdrawal and intensive care treatment. Thus, these patients received ASC under compassionate use. Other treatments performed are given in Table 3.

Clinical course after treatment with ASC

Clinical situation after treatment with ASC improved in all patients over different time courses (Table 4). As objective measure for liver injury, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured multiple times before and after treatment. AST and ALT serum levels were strongly elevated before ASC treatment (AST: 412 ± 153 U/L; ALT: 158 ± 127 U/L; Figure 1a,b). After treatment with ASC, AST and ALT concentrations dropped to normal levels within 25 days (case II), 88 days (case I), or 59 days (case III). Measures of coagulation (Quick, international normalized ratio; Figure 2a,b) were not altered in these patients; thus, no improvement by ASC treatment could be observed. This might be due to vitamin K

Table 1. Characteristics of the adipose-derived stem cells used for treatment under compassionate use

	Passage	Viability/%	CD90+/%	CD45+/%	CD34+/%	CD105+/%	HLA-II +/%	CD73+/%
Donor A	0	93.2	97	0	1	83	0	94
	1	95.3	98	1	1	95	0	97
	2	97.0	98	0	0	92	0	96
	3	95.0	99	0	0	95	0	98
	4	95.1	98	0	0	92	0	96
	5	97.2	96	0	1	91	0	95
Donor B	0-part1 ^a	94.4	97	0	5	90	0	97
	0-part2 ^a	95.6	97	0	17	79	1	96
	1	95.5	97	0	2	94	1	96
	2	95.6	94	0	3	88	1	92

Cell viability and immune phenotype of the ASC during cultivations were determined. The ASC criteria as described by (Bourin et al. (14)): viability \geq 90%; CD90⁺ \geq 80%; CD105⁺ \geq 80%; CD73⁺ \geq 80%; CD34⁺ variable up to 30%; CD45⁺ \leq 2%; HLA II⁺ no recommendation but should not be higher than \leq 2%.
HLA, human leukocyte antigen.
^aPassage 0 of donor B was divided into 2 parts.

dosage for 3 days after admission in cases II and III. The model for end-stage liver disease (MELD) score, used to assess severity of liver disease and used for organ allocation at Eurotransplant (15), was initially at moderate to high values (15–23 points). At discharge after administration of ASC, the MELD was clearly reduced in all cases (10–16 points). Transient elastography was available for cases II and III before and after treatment with ASC. The latest measurements before treatment were at 22 and 17.3 kPa, respectively, which would indicate cirrhosis in chronic liver injury (Figure 3a). It has been shown that acute liver injury also leads to an increase of liver stiffness, as does the excessive collagen deposition in cirrhosis (16–18). These high values for liver stiffness thus may be interpreted as severe acute injury. After treatment with ASC in both cases, a strong reduction of liver stiffness was observable, with case III reaching normal values 75 days after treatment. Case II had normal transient elastography readings 60 days after treatment. For the same cases, LiMax measurements (methacetin C¹³ breath test) were available demonstrating impaired liver function for cases II and III on or directly before the date of ASC treatment (Figure 3b). After treatment, case II exhibited normal liver function according to LiMax measurements within 35 days, case III within 88 days, respectively.

Duration of hospitalization

Total time of hospitalization for case I was 32 days, after first treatment with ASC hospitalization continued for 27 days before discharge in stable condition. For case II, hospitalization lasted 15 days, and after first ASC dosage, the stay lasted 14 days

before discharge in improved general condition. Case III was hospitalized for a total of 9 days and 6 days after first treatment with ASC.

Case I was readmitted 1 month after the initial hospital stay, as ascites was progressing. Diuretic measures were taken, and diuretic medication was adapted, resulting in reduction of ascites. Furthermore, a gastrointestinal bleeding occurred from varices, which could be stopped by endoscopic intervention. After 10 days of hospitalization, the patient was discharged.

Comparison to historic data

Owing to the nature of investigational compassionate use of therapeutic interventions, no controls or randomization is possible. To give a rough estimate for efficacy of ASC treatment, data of the 3 cases were put into relation with historical data. One study used as point of reference investigated 61 patients with alcoholic hepatitis and demonstrated superiority of prednisolone treatment for 2-month mortality (19). In this study, bilirubin and prothrombin time (quick) was available over a time course of 2 months in weekly intervals. In Figure 4, the course of serum bilirubin serum concentrations and quick of 3 cases under ASC treatment are compared with data from the study by Ramond et al. Day 0 refers to start of ASC treatment for cases or randomization for historical data. Bilirubin values of presented cases at start of therapy varied to great extent, although the reduction of bilirubin serum concentrations was significantly faster than in patients of the study by Ramond et al. (Figure 4a). Prothrombin time was better in all cases than in the

Table 2. Basic patient characteristics

Case number	Sex	Age	General condition	CHILD class	Ascites	Jaundice	Liver steatosis
I	Female	59	Considerably impaired	C	Yes (moderate)	Yes (skin and sclera)	Yes and hepatomegaly
II	Female	43	Impaired	B	No	Yes (skin and sclera)	Yes
III	Male	43	Impaired	B	No	Yes (mild)	Yes

Table 3. ASC treatment

Case number	Start of treatment after hospital admission	No. of treatments	Other medication
I	5 d	6	Torsemid and spironolactone
II	1 d	4	Liquid infusion rifaximin, vitamin K, and ceftriaxon (for infection of respiratory tract)
III	10 d	2	Liquid infusion, vitamin K, and LOLA (for hepatic encephalopathy)

cohort of Ramond et al. at the start of therapy. Nevertheless, cases under ASC treatment exhibited a clear increase of quick percentage, while prednisolone therapy in historical cases only led to mild improvement over 2 months. As reference point for the course of liver stiffness, historical data from 34 patients with acute hepatitis B virus (HBV) infection were used (20). Data of the cases under ASC treatment (2 with available liver stiffness measurements) are superimposed on historical data (Figure 4c). Stiffness at start of therapy or shortly before was above the 75th percentile of historical patients with acute HBV. Four weeks after ASC treatment, the liver stiffness was close to 25th percentile of acute HBV patients after 4 weeks. Relating clinical data of cases under ASC treatment to historical data from patients with alcoholic hepatitis or acute HBV indicates a relatively fast recovery of bilirubin, coagulation, and liver stiffness under ASC therapy.

DISCUSSION

In the treatment of patients with LF, a lot of progress has been achieved, and by intensive care measures and LT as final option, many patients can be rescued from this severe and life-threatening condition (21). However, there are still a relevant number of cases, where intensive care treatment is not sufficient or where etiology does not allow specialized treatment option (22). In particular, active use of alcohol or drugs prohibits LT, leaving many patients without treatment options in

this grave condition. In the present case report, we demonstrate that infusion of mesenchymal stem cells, in this particular case ASC, can on the one hand support recovery from LF and is safe.

All presented cases were due to or associated to alcohol intake, with known chronic abuse in 2 cases. Cases I and II presented with signs of AOCLF, with case 1 demonstrating significantly reduced general condition, at the time of admission to the Department of Gastroenterology, Hepatology, and Infectious Diseases. One limitation of the available records is that the LF process in all cases had been going on for an unknown time before admission to our hospital as all of them were referred from secondary care hospitals or a specialist practitioner. During the stay at intensive care ward, no relevant improvement in condition was observed, despite cessation of alcohol consumption and medical support to current standards.

On treatment with ASC under compassionate use, all 3 cases exhibited improvement of the general condition and recovery of indicators for liver injury as transaminase serum levels, liver stiffness, and liver function assessed by the breath test. The duration for recovery of these parameters was different between individuals and also between parameters. This is also related to another limitation, since not all measurements were performed in parallel and in the same rhythm. For example, liver function by the methacetin breath test was only available for 2 cases before treatment and 2 or 3 months after treatment, respectively. Thus, an actually faster recovery of liver function could be possible, which cannot be demonstrated by available data. In general, clinical experience suggests that recovery from the LF in all cases, in particular in case I with AOCLF, presenting in significantly impaired general condition, allowing hospital discharge within a time frame of 1 month seems quite rapid. Recovery of case III until discharge was less than 1 week after ASC treatment. Clinical management and general anamnesis thus would suggest a support of liver regeneration by ASC treatment. Since no randomization or control population was recruited in this compassionate use setting, historical data were compared with available data of the 3 cases. In comparison with a cohort of 60 individuals with alcohol-induced ALF under prednisolone treatment, reduction of bilirubin was similar or better (comparing the slope of decline), and improvement of the quick was better, albeit with better initial values of our cases at presentation (19). For the course of liver stiffness, no relevant

Table 4. Individual serum measurements before and after treatment with ASC

Parameter	Case I at first treatment	Case I 8 d after treatment	Case II at first treatment	Case II 6 d after treatment	Case III at first treatment	Case III 6 d after treatment
AST	239.2	169.8	368.4	320.2	491.8	242.7
ALT	144	101.7	38.2	47.6	290.3	142.8
γGT	79.2	82.2 (after 15 d)	798	541.8 (after 7 d)	5,436	2,749.8
Bilirubin	506.4	265.2	117.7	51.3	112.2	44.3
Creatinine	46	64 (at discharge)	47	50 (at discharge)	82	88 (at discharge)
INR	1.1	1.1	1.1	1.2	1.1	0.98
MELD	23	16 (at discharge)	15	11 (at discharge)	14	10 (at discharge)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γGT, gamma glutamyltransferase; INR, international normalized ratio, MELD, model for end-stage liver disease.

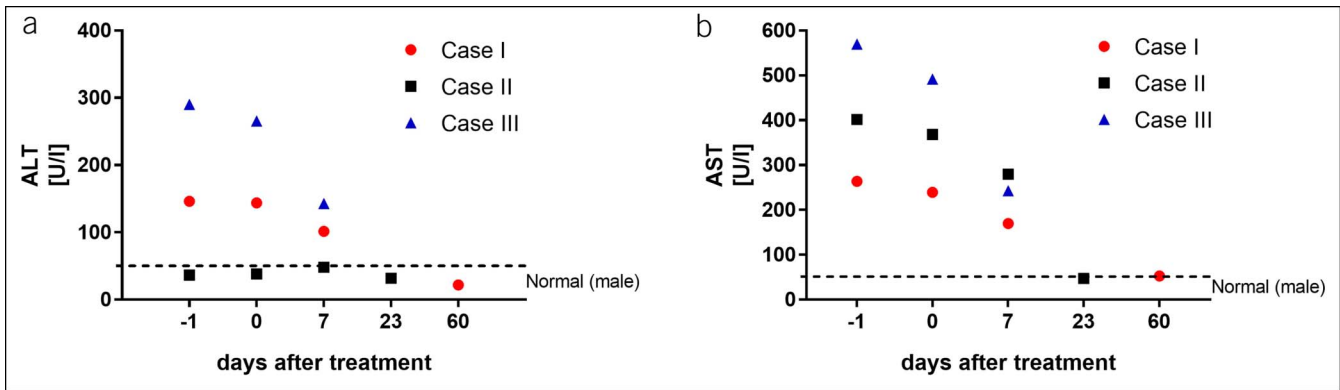


Figure 1. Course of serum transaminase concentrations in patients with alcohol-induced liver failure and ASC treatment. ALT(a) and AST(b) serum concentrations were drastically elevated above normal ranges in all 3 cases, except for ALT in case II. After treatment with ASC, a reduction of both ALT and AST was observed, reaching normal ranges within 1–2 month in all cases. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

historical control group with alcohol-induced ALF or AOCLF could be identified. When comparing the course of liver stiffness with 34 historical patients with acute HBV (acute hepatitis but no LF) (20), initial liver stiffness of the cases was around the 75th percentile of acute HBV patients. After treatment with ASC, LS dropped in the cases to below the 25th percentile of the patients with acute HBV within 4 weeks, suggesting a very quick return to a normal liver density or restoration of pressure. Notably creatinine levels were not affected by ASC treatment, resulting in only a slight reduction of MELD until discharge. Further studies into the mechanisms of ASC might be able to explain the differential effects on LS and MELD value. The collected data on patients with alcohol-induced acute (on-chronic) LF with ASC treatment seem to support that ASC treatment can facilitate timely recovery from an LF event.

Follow-up for the 3 cases is currently continued with the latest data deriving from 56, 75, and 88 days after treatment, respectively. All cases survived up to now and did not show any signs of adverse effects. Case I was rehospitalized 1 month after the initial stay due to expanding ascites. By adapting diuretic measures and release of liquid from the abdomen, this could be

controlled without further complication. We believe that this condition was not related to ASC treatment but rather derives from the extent of liver injury from intensive long-term alcohol abuse. However, it cannot be excluded with absolute certainty that ASC treatment might have influenced expansion of ascites. Further studies will need to clarify, if this event was unrelated to treatment—as we believe—or might be connected. Overall, ASC treatment for LF events seems safe and did not lead to adverse events in the presented cases.

There are several limitations to this case series. Owing to use of ASC under investigational compassionate use, randomization or any other recruitment of fitting control patients was prohibited. Time points for measurements of clinical parameters could not be predetermined under investigational compassionate use, limiting the comparability of data between cases. Finally, the potential mechanisms underlying the effect of ASC infusion on LF symptoms could not be elucidated. From preclinical data of other groups, we infer humoral and paracrine factors as main mediators for the effect of ASC treatment (7,8,10,23–29). This factor might be a single cytokine, i.e., interleukin-10, or growth factor, i.e., vascular endothelia growth factor, or could be the composition of the secretome of ASC as a whole. Further clinical studies and

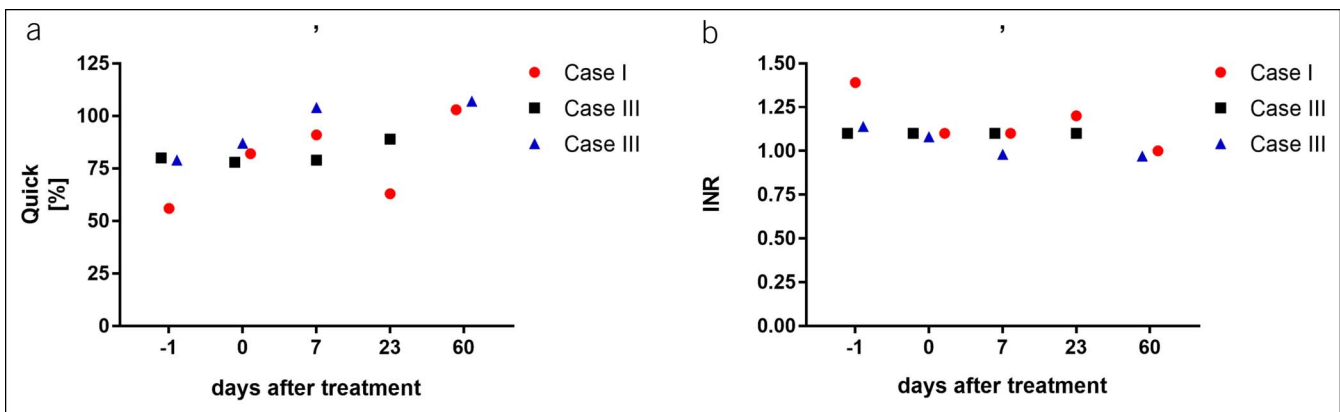


Figure 2. No significant alteration of coagulation measures in alcohol-induced liver failure. All cases exhibited only a slightly reduced quick (a), which reached normal values after 1 or 2 months after ASC treatment. INR (b) was increased in 2 cases, dropping to normal values around 1 week after ASC treatment. INR, international normalized ratio.

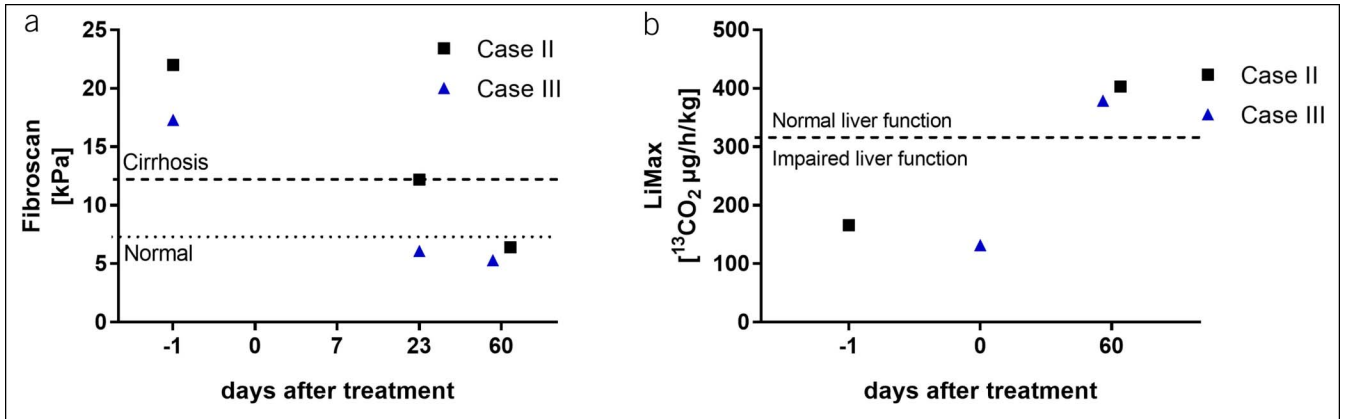


Figure 3. Liver stiffness and liver function tests indicate rapid recovery from liver failure after treatment with ASC. In 2 cases, liver stiffness detected by transient elastography and liver function measured by the methacetin breath test were available. Liver stiffness (a) was strongly elevated in both cases but dropped to normal values after 23 days or 2 months after ASC treatment, respectively. Liver function (b) by the breath test was impaired at admission and reached normal function 2 months after ASC treatment in both cases (no earlier time points available).

experiments are required to identify the probably secreted factors mediating improvement of liver function or regeneration.

In summary, we present 3 cases with alcohol-induced ALF or ACLF, who received ASC treatments under compassionate use.

Clinical data suggest that ASC treatment can enhance recovery from ALF or AOCLF events and is safe. Subsequent clinical studies are required to demonstrate an unequivocal supportive effect of ASC treatment for LF.

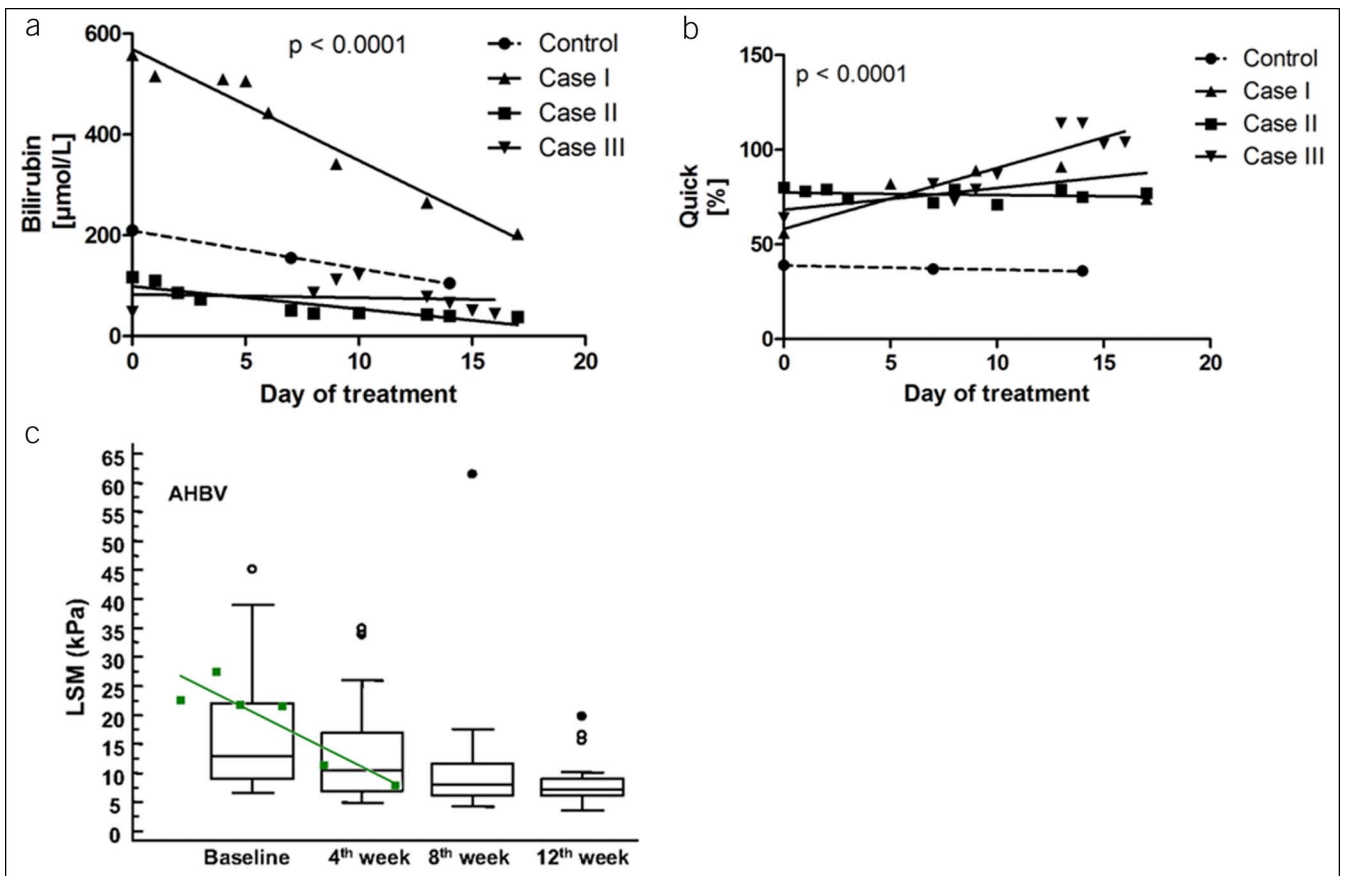


Figure 4. Comparison of cases under ASC treatment to historical data. Bilirubin (a) and prothrombin time (quick %; b) from a cohort of 61 patients with alcoholic hepatitis and prednisolone therapy (19) are given in parallel to data from the 3 presented cases. Bilirubin reduction and increase of quick were significantly steeper in cases than in historical patients. However, prothrombin time was initially diminished to a greater extent in historical patients. Liver stiffness measurements (c) of 34 historical patients with acute HBV (20) are compared with data from cases II and III. Cases had liver stiffness in the uppermost percentile of HBV patients before ASC treatment, which dropped to the 25th percentile 4 weeks after treatment. HBV, hepatitis B virus infection

CONFLICTS OF INTEREST

Guarantor of the article: Ali Canbay, MD.

Specific author contributions: M.K., J.M., and A.C.: study concept and design. T.G., J.M., and M.D., S.B.: acquisition of data. J.M.: supply of resources and administrative support. S.B. and J.P.S.: analysis and interpretation of data. M.D., S.B., and T.G.: drafting of the manuscript. M.K., J.P.S., and A.C.: critical revision of the manuscript for important intellectual content. J.P.S.: statistical analysis. M.K.: obtained funding. M.K. and A.C.: study supervision. All authors approved the final draft submitted.

Financial support: None to report.

Potential competing interests: M.K., J.M., M.D., and S.B. are/were directly associated with Oxacell AG. T.G., J.P.S., and A.C. declare that they have no conflicts of interest.

Study Highlights**WHAT IS KNOWN**

- ✓ LF has limited treatment options.
- ✓ Enhancing liver regeneration might be feasible to assist recovery from LF.
- ✓ ASC are promising candidates for such treatment.

WHAT IS NEW HERE

- ✓ Treatment with ASC for LF seems to be safe.
- ✓ ASC treatment seems to accelerate recovery from LF.

TRANSLATIONAL IMPACT

- ✓ In cases of LF due to long- and/or short-term alcohol abuse, treatment with ASC might assist in recovery.

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