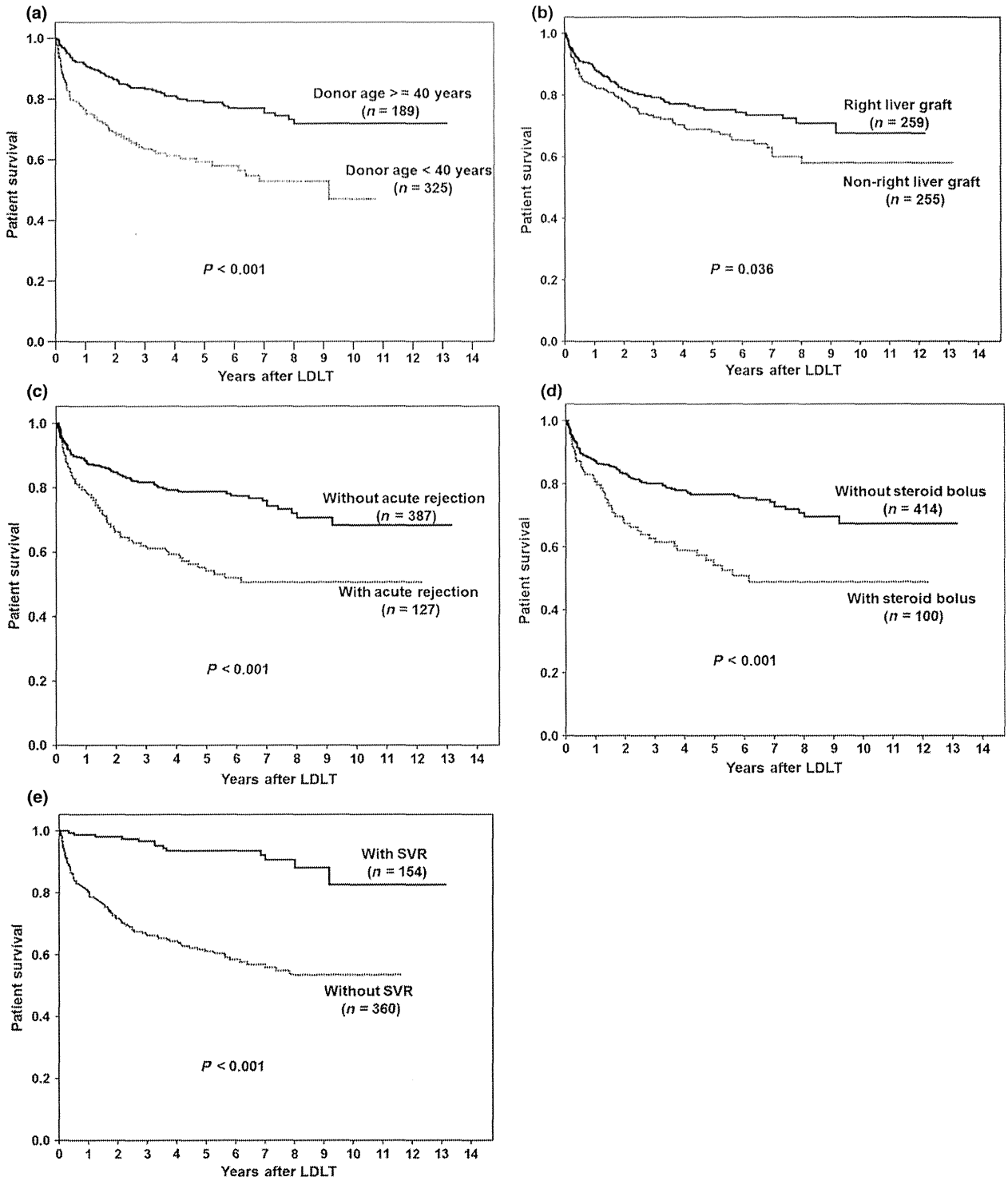

Figure 2 Kaplan–Meier curves stratified by each variable: (a) donor age, (b) graft type, (c) acute rejection, (d) steroid bolus, and (e) sustained virologic response. *LDLT, living donor liver transplantation; SVR, sustained virologic response.*

increased use of liver grafts from older donors. For HCV-positive recipients, two large retrospective reports from the Scientific Registry of Transplant Recipients and UNOS databases reported that donor age over 40 is an independent predictor of patient death [15,16]. Other accumulating reports [14,17,18] indicate that the grafts from older

Table 4. Factors associated with patient survival among those achieved SVR (n = 154).

Cox regression analysis	Hazard ratio (95% confidence interval)	P-value
Recipient age: ≥60 years (n = 43) vs. <60 years (n = 111)	1.424 (0.318–2.385)	0.644
Recipient gender: male (n = 100) versus female (n = 54)	4.709 (0.918–24.161)	0.063
Pretransplant antiviral treatment: yes (n = 66) versus no (n = 88)	1.666 (0.350–7.931)	0.522
HCV genotype: 1b (n = 112) versus other types (n = 42)	0.873 (0.203–3.747)	0.855
Co-existence of HCC: yes (n = 54) versus no (n = 100)	0.728 (0.179–2.694)	0.635
MELD score: ≥15 (n = 54) vs. <15 (n = 98)	1.354 (0.578–3.204)	0.785
LDLT cases per year: ≥20 (n = 82) vs. <20 (n = 72)	1.054 (0.458–1.254)	0.854
Calcineurin inhibitor: Tac (n = 94) versus CyA (n = 60)	3.580 (0.736–17.421)	0.114
Mycophenolate mofetil: yes (n = 78) versus no (n = 76)	0.932 (0.456–1.884)	0.781
Steroid withdrawal: yes (n = 40) versus no (n = 114)	0.449 (0.096–2.102)	0.31
Splenectomy: yes (n = 59) versus no (n = 95)	1.402 (0.335–5.873)	0.644
Episode of acute rejection: yes (n = 34) versus no (n = 120)	1.854 (0.216–15.914)	0.574
Steroid bolus injection: yes (n = 26) versus no (n = 128)	0.16 (0.019–1.386)	0.096
Donor age: ≥40 years (n = 43) vs. <40 years (n = 111)	1.18 (0.296–4.698)	0.815
Type of graft: right liver (n = 80) versus non-right liver (n = 74)	2.799 (0.818–9.573)	0.101

HCV, hepatitis C virus; HCC, hepatocellular carcinoma; LDLT, living donor liver transplantation; MELD, model for end-stage liver disease; Tac, tacrolimus; CsA, cyclosporine; SVR, sustained virologic response.

Table 5. Summary of antiviral treatment.

	Total (n = 361)	Treatment for established recurrent hepatitis C (n = 211)	Preemptive treatment (n = 150)
Time since LDLT (months)	3 (0–102)	4 (0.5–102)	1 (0–68)
Treatment duration (months)	15 (0.3–99)	14 (0.3–99)	17 (0.3–55)
Regimen: PEG-INF alfa-2a/RBV	45 (12%)	33 (16%)	12 (8%)
PEG-INF alfa-2b/RBV	223 (62%)	146 (69%)	77 (51%)
INF alfa-2b	93 (26%)	32 (15%)	61 (41%)
Dose reduction	143 (40%)	85 (40%)	58 (39%)
Discontinuation	150 (42%)	66 (31%)	84 (56%)
Sustained virologic response	154 (43%)	89 (42%)	65 (43%)

LDLT, living donor liver transplantation; PEG-INF, pegylated-interferon; RBV, ribavirin; INF, interferon.

donors are at greater risk for disease progression and impaired graft/patient survival compared with those from younger donors. Our results are definitely consistent with these reports.

Acute rejection in conjunction with treatment with a steroid bolus is one of the most critical factors to address with respect to HCV recurrence. Historical studies [19,20] have demonstrated that steroid bolus for acute rejection in HCV-positive recipients accelerates the recurrence of hepatitis and decreases patient survival. A recent study reported that HCV-positive recipients who receive high-dose steroid treatment for acute rejection are at increased risk of severe recurrent hepatitis, in which older donor age and an episode of rejection are the two most important predictors of developing fibrosing cholestatic hepatitis [21]. Similarly, our study also revealed that both older donor age and acute rejection are independent predictors for impaired patient outcome among LDLT recipients.

The association between achieving SVR and graft/patient survival after liver transplantation for HCV-positive recipients is a matter of debate [10]. Many studies with standard dual treatment of PEG-INF/RBV for 12 months in a DDLT setting have implied a survival benefit of achieving SVR [8,22], but there has been no evidence to support the recommendation of antiviral treatment for recurrent graft hepatitis C due to the lack of clinical benefit with sufficient long-term observation and the existence of frequent severe adverse effects, as concluded by a recent Cochrane meta-analysis [10]. Recent retrospective cohort studies with a long follow-up duration reported improved patient/graft survival in patients who obtained an SVR after antiviral treatment [23–25]. In accordance with those reports, our retrospective analysis indicated a positive effect of achieving SVR on patient survival. Caution should be taken in interpreting our results; however, as SVR was assessed among the whole cohort, including patients who were not indicated for antiviral treatment, the follow-up period after achieving SVR was rather short, and most importantly, a large variety of antiviral treatment regimens were used in Japan, which will be described later.

A noteworthy finding in the present retrospective analysis is the impaired patient survival in recipients who received a non-right liver graft (left liver in 239 cases and right lateral sector in 16 cases). Recent studies comparing outcomes between LDLT and DDLT in HCV-positive recipients have reported equal or even improved outcomes both in patient/graft survival and in fibrosis progression in the LDLT setting, which could be attributed to the younger donor age and shorter ischemic time of LDLT grafts [13,14,26–29].

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Based on these findings, LDLT for HCV-positive recipients is now widely accepted as an established alternative to DDLT, even in Western countries. On the contrary, however, the present finding may raise an alarm for reduced size grafts, as a left or posterior graft is clearly smaller than a right liver graft. Another point to be emphasized here is that all LDLTs investigated in the aforementioned studies comparing LDLT and DDLT were universally performed with right liver grafts. One possible explanation for the inferior outcome of the smaller graft is that the intense hepatocyte proliferation that occurs in smaller partial liver grafts may lead to increased viral translation and replication, as advocated by previous authors [30–32]. However, there are several limitations among these speculations. First, the data of the viral load, which is reported to reach a maximum level between the first and third post-transplant months [33], were not available in this study to demonstrate the higher viral replication in the smaller grafts during this period. Another is that the graft type selection is based on the ratio of the volume of the graft to recipient body weight or standard liver volume in our society, which will lead to the bias in the comparison of the right liver versus non-right liver graft. Despite these limitations, considering that comparable outcomes between left liver graft and right liver graft have been reported by us [34] and others [35] in LDLT recipients as a whole, caution should be taken in selecting the type of graft (left versus right) for HCV-positive recipients. Thus, future LDLT studies are required to investigate whether a smaller partial liver graft (left liver) is potentially inferior compared with a larger graft (right liver) in terms of graft/patient survival and recurrent hepatitis severity among HCV-positive recipients.

The antiviral treatment for recurrent hepatitis C after LDLT in Japan was also reviewed in the present study. As described elsewhere in detail [11], the antiviral treatment regimen in Japan differs widely from center to center; preemptive treatment versus treatment after confirmation of recurrent disease, starting dose and method of escalation, and the duration of treatment (usually longer than 12 months). Consequently, our data only present an overview of antiviral treatment in Japan, and no definite conclusion can be drawn regarding the actual efficacy of antiviral treatment after LDLT. Moreover, based on the recent prospective, multicenter, randomized study by Bzowej *et al.* [36], European and USA transplant societies do not support the routine use of preemptive antiviral therapy. A review of Western literature regarding the standard 12-month PEG-INF/RBV treatment for established recurrent hepatitis C after DDLT reveals that the median SVR rate is 33% (0–56%) with a dose reduction rate of 70% and a discontinuation rate of 30% [37]. The present result of an SVR rate of 43% with a dose reduction rate of 40% and a discontinuation rate of 42% seems not so different from those of previous literatures; however, as discussed above, the diversity in the methods, the doses, and the duration of treatment in Japan preclude the direct comparison with Western findings.

Conclusion

This retrospective analysis of the largest series of LDLT for HCV-positive recipients in Japan revealed 5- and 10-year survival rates of 72% and 63%, respectively, and that donor age (>40), non-right liver graft, an acute rejection episode, and the absence of SVR are independent predictors of patient survival. Based on the present result, caution should be made in the selection of the left liver graft for HCV-positive recipients; however, the development of more effective antiviral treatment in the near future may facilitate the application of the left liver graft.

Authorship

YM: designed the study. TI: collected data. NA, YS, NK, SE, TF, HO, HN, AT, YK, MS, YK, KY, KS, MM and MT: performed the study. NA and YS: analyzed and wrote the paper.

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ORIGINAL ARTICLE

Use of simeprevir following pre-emptive pegylated interferon/ribavirin treatment for recurrent hepatitis C in living donor liver transplant recipients: a 12-week pilot study

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Abstract
Background The management of recurrent hepatitis C following liver transplantation remains a challenge.
Methods We prospectively investigated the efficacy and safety of simeprevir in combination with pegylated interferon and ribavirin in five patients undergoing living donor liver transplantation (LDLT) with recurrent hepatitis due to hepatitis C virus (HCV) genotype 1b.
Results As the immunosuppressive regimen, four received cyclosporine A (CsA) and one received tacrolimus (FK); no dose adjustment was made prior to the introduction of simeprevir, but the dose was accordingly modified afterwards. All five patients completed the intended 12-week treatment course without significant adverse events greater than grade 2, and no episodes of rejection were detected during the study period. The trough levels of CsA and FK were stably maintained. At week 12, HCV-RNA was not detectable in three of the five patients, whereas the HCV titer of the other two patients, including one with Q80L and V170I mutations at the HCV NS3 position, was at the lower level of quantification (1.2 $\log_{10}$ IU/ml).
Conclusions Based on this pilot study, simeprevir-based triple therapy is safe and somewhat effective within the first 12 weeks in LDLT recipients with HCV recurrence. Further studies are warranted to obtain robust conclusions.

Keywords Direct-acting antiviral drugs · Hepatitis C · Living donor liver transplantation · Simeprevir

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Introduction

Compared with liver transplant patients not infected with hepatitis C virus (HCV), those with HCV have a poorer post-transplant prognosis [1–3], especially when the virologic response is inadequate [4, 5]. The lower antiviral response in liver transplant recipients, however, limits the efficacy of conventional interferon-based antiviral treatment (pegylated interferon [Peg-IFN] and ribavirin [RBV]) for recurrent hepatitis C following liver transplantation [6].

In the past several years, the development of direct-acting antiviral drugs (DAA), telaprevir (TVR) and boceprevir (BOC), for the treatment of HCV genotype 1 has provided a promising treatment option [7, 8]. Although the feasible efficacy of triple therapy, including such "1st generation protease inhibitors", has been demonstrated by several groups, the likelihood and severity of adverse events seem to be inevitable and have limited its use as the first choice for recurrent hepatitis C post-liver transplantation [9]. In addition, it is difficult to maintain the levels of calcineurin inhibitors such as cyclosporine A (CsA) or tacrolimus (FK)

in combination with the 1st generation DAA, which are primarily metabolized by the cytochrome P450 3A4 pathway [10].

In December 2013, simeprevir (SMV), which is a one-pill, once-daily, oral HCV NS3/4A protease inhibitor, a so-called "2nd generation protease inhibitor", was approved for clinical use in Japan. SMV is associated with few adverse events, but the antiviral effects in patients with hepatitis C are as good or better than those of DAA [8]. In liver transplant recipients, SMV is likely superior to prior DAAs in terms of drug interactions, based on its small impact on the blood levels of calcineurin inhibitors when used simultaneously [10].

We conducted this prospective pilot study to evaluate the feasibility of SMV-based triple therapy in liver transplant recipients with hepatitis C, mainly with respect to the antiviral response, adverse events, and drug interactions with immunosuppressants by week 12 (namely by the cessation of SMV).

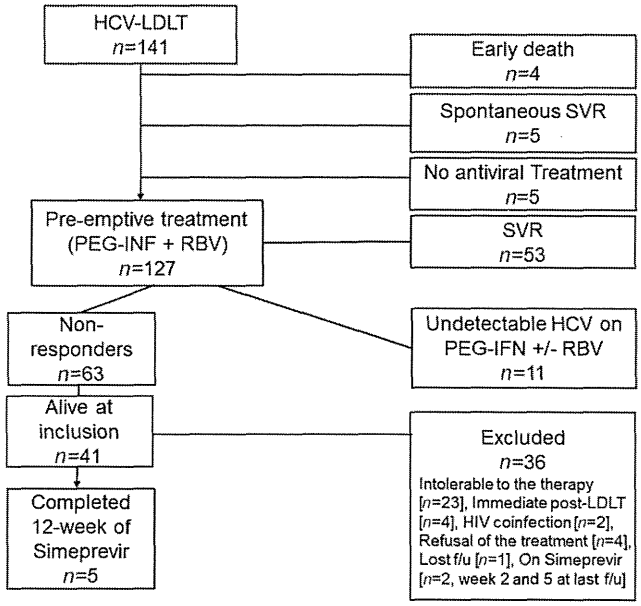


Fig. 1 Flow diagram of the patients enrolled in the simeprevir-based triple therapy

Materials and methods

Antiviral treatment regimen and patient selection

Between January 1996 and December 2013, 141 adult-to-adult living donor liver transplantations (LDLTs) were performed for HCV-positive recipients at the University of Tokyo Hospital. As previously reported [11], antiviral treatment was generally initiated with low-dose Peg-IFN alpha-2b and RBV 200–400 mg/day promptly after improvement of the general condition following liver transplantation in our institution. Recovery of hematologic and renal function was considered crucial, with a leukocyte number >4000/ml, platelet count >50000/ml, hemoglobin >8 g/l, and serum creatinine levels <2 mg/dl. During conventional dual treatment, flexible dose adjustments were made as necessary to avoid serious adverse events. A fixed overall treatment period length was not defined. Splenectomy was performed at the time of LDLT to prevent the progression of thrombocytopenia under IFN-based antiviral therapy [12].

Pre-emptive Peg-IFN /RBV treatment was administered to 127 of our 141 HCV-positive LDLT recipients, excluding cases of early death (within 3 months) after LDLT ($n = 4$), cases with spontaneous sustained virologic response (SVR) ($n = 5$), and cases without antiviral treatment due to clinical decision ($n = 5$). SVR was achieved in 53 patients, 11 had undetectable HCV-RNA on Peg-IFN and RBV therapy (dual treatment) upon inclusion; the remaining 63 were classified as non-responders. We selected patients for the current study among the 41 non-responders who were alive with sustainably positive HCV-RNA at the time of inclusion in this study. Patients who had either not tolerated or were not expected to tolerate conventional dual treatment were excluded. The current study protocol was not intended for those who were immediately post-transplant or were coinfected with human immunodeficiency virus (HIV) because of the lack of a detailed profile of SMV-based triple therapy in the transplant setting, considering the risk of unknown adverse events that could be fatal in this population, but only for those who survived the perioperative period and tolerated dual therapy for recurrent hepatitis C. Patient selection is shown in the flowchart in Figure 1.

SMV (100 mg daily) was intended to be continued for 12 weeks in combination with Peg-IFN and RBV (triple-antiviral treatment), followed by 36 weeks of dual treatment. The patients were generally admitted for 1 week, both to undergo liver biopsy pre-induction of SMV and to carefully monitor the daily change in the trough levels of calcineurin inhibitors (CNIs) following the induction of SMV.

Here we prospectively studied the 12-week clinical courses of all five patients who met the inclusion criteria and in whom triple-antiviral therapy with SMV was initiated by the end of March 2014, and followed up by the end of June 2014.

Laboratory test and histopathology assessment

Conventional blood work for the management of the patients with post-transplant hepatitis was checked as necessary. The estimated glomerular filtration rate

(eGFR; ml/min per 1.73 m^2) was calculated using the following formula: 194 × serum creatinine (−1.094) × age (−0.287) × 0.739 (if female), Japanese equation (equation 4) [13]. HCV RNA was measured quantitatively by reverse-transcriptase polymerase chain reaction (Amplicor HCV; Roche Molecular Systems, Pleasanton, CA, USA). Before liver transplantation, the HCV genotype was determined: the HCV genotype in all the five patients was 1b. In addition, the nucleotide sequences of the core and the number of amino acid substitutions in the interferon sensitivity-determining region (ISDR) in the NS5A gene were determined using a direct sequencing method [14]. The interleukin 28B (IL28B) genotype rs8099917 was also examined using the Invader assay (Third Wave Technologies, Madison, WI, USA) [15]. Prior to the induction of SMV, HCV NS3 and NS5A sequencing was determined, and liver biopsy was performed and evaluated by a pathologist based on the Metavir score [16].

Immunosuppression

Our post-transplant strategy for immunosuppression is documented elsewhere [11, 17]: briefly, it comprises steroid induction with CsA or FK, and the doses of each drug are gradually tapered for 6 months after LDLT. Methylprednisolone is tapered from 3 mg/kg on the first postoperative day to 0.05 mg/kg at the sixth postoperative month, and a maintenance dose of 2–4 mg of methylprednisolone is continued in all recipients. Mycophenolate mofetil (MMF) is added mainly for recipients requiring CNI dose reduction.

Ethics statement

The study protocol was approved as project number 2032, and human subject research regarding the IL28 polymorphism was particularly approved as project number G3514 by the Graduate School of Medicine and Faculty of Medicine at the University of Tokyo Research Ethics Committee; and the Human Genome, Gene Analysis Research Ethics Committee.

Statistical analysis

We used SPSS 17.0 statistical software (SPSS, Chicago, IL, USA) to analyze the relevant data. Differences between groups were analyzed by the Mann–Whitney *U*-test or ANOVA for continuous variables as appropriate, and the χ^2 test for categorical variables. *P*-values <0.05 were considered significant.

Results

The clinical characteristics of those five LDLT recipients are shown in Table 1. The median Model for End-Stage Liver Disease score was 15 (range 9–23). None of the five was coinfected with HIV, and four (80%) had hepatocellular carcinoma within the Milan criteria [18]. The details of each patient, including the HCV profile and the single nucleotide polymorphisms of IL28B rs8099917, are shown in Table 1. The Q80L/V170I and S122T/V170 mutations in NS3 were detected in patient #2 and 3, respectively. Q54H, F37L, Q54H, F37L/Q54H/Q62E, F37L mutations in NS5A were detected in patient #1 to 5, respectively.

Efficacy

All five patients completed the 12-week course of triple therapy with SMV. All of them were treated with dual therapy with Peg-IFN and RBV afterward.

Three of the five patients achieved an undetectable viral load of HCV at week 4, 8, and 12 weeks, and the viral titer of the remaining two patients was at the lower level of quantification (LLOQ, <1.2 $\log_{10}$ IU/ml) at week 4; one patient achieved an undetectable viral load at week 8, but the viral load became detectable again at week 12. The HCV titer of the remaining patient remained around LLOQ at weeks 8 and 12 (Table 1). At the last follow up (median 22 [range 16–27] weeks since the initiation of triple therapy), HCV viral load of those with undetectable HCV-RNA at week 12 were sustained to be below detectable level, although those with positive HCV-RNA at week 12 were both positive then (1.4 and 7.5 $\log_{10}$ IU/ml). HCV-RNA levels in the five patients are shown in Figure 2.

Safety profile and immunosuppression levels with SMV

No significant adverse events were observed other than grade 2 diarrhea in patient #1 on day 26, which was resolved immediately (within 1 week) after the reduction of mycophenolate mofetil (MMF) from 3000 mg/day to 1500 mg/day. None of the five patients required a dose reduction of Peg-IFN or RBV, use of granulocyte-colony stimulating factor for neutropenia, or blood transfusion for anemia. Renal function was well preserved during the study period, with no significant change in eGFR before or after the introduction of SMV (median 68 [range, 39.1–97.2] to 64.9 [range, 44.5–102] ml/min, $P = 0.84$). Bilirubin levels were not increased in any of the five patients. Immunosuppression was not modified before the initiation of SMV. The CsA trough levels before (median 78 [range 48–113] ng/ml), 1 week after (median 68.5 [67–104] ng/ml) and 12 weeks after (median 72.5 [65–92] ng/ml) initiating the triple therapy did not differ significantly ($P = 0.72$), and the FK

Table 1 Patient characteristics

Patient #	1	2	3	4	5
Age (years)	51	64	66	49	59
Sex	M	F	M	M	F
Height (cm) / weight (kg)	170/65	147/54	166/56	168/63	156/53
Donor age (years)	50	30	24	44	60
Donor relationship	Spouse	Daughter	Son	Spouse	Spouse
Calcineurin inhibitor (mg/day)	CsA (40)	CsA (75)	CsA (60)	FK (2)	CsA (60)
MMF (mg/day)	3000	None	1000	1500	None
Histopathological activity and fibrosis at triple therapy[a]	A2 / F1	A0-1 / F0-1	A1 / F1	A0 / F0	A1 / F1
Baseline clinical chemistry at triple therapy					
Total bilirubin (mg/dl)	1.9	0.8	0.9	0.9	0.7
Alanine aminotransferase (IU/ml)	68	31	47	25	29
Creatinine (mg/dl) and Estimated GFR (ml/min)	0.65 / 100.5	0.64 / 70.5	1.43 / 39.4	1.33 / 46.2	0.61 / 76
International normalized ratio	1.29 (on warfarin)	0.90	0.85	0.95	0.84
Hemoglobin (g/dl)	9.0	8.5	12.3	13.5	9.6
Leukocytes (/ul)	5900	5000	4900	5900	4600
Platelets (/ul)	476000	145000	186000	192000	262000
NS3 mutation	Non	Q80L/V170I	S122T/V170I	Non	Non
NS5A mutation	Q54H	F37L	Q54H	F37L/Q54H/Q62E	F37L
Pre-transplant antiviral therapy	Relapse	Non responder	Not applicable	Not applicable	Not applicable
Baseline HCV-RNA pre-LT (log_{10} IU/ml)	3.1	6.4	7.1	6.7	5.7
TPV therapy post -LT	Relapse	Not applicable	Relapse	Not applicable	Not applicable
Pre-triple treatment interferon (mo) since LT	23	16	118	26	16
Dose of Peg-IFN α2b (μg/week)	80	70	100	100	100
RBV dose (mg/day)	200	200	200	200	200
%CNI after the triple therapy	50%	67%	100%	75%	100%
CNI trough at triple therapy (ng/ml)	113	73	48	9.8	83
CNI trough 1 week after initiation (ng/ml)	104	69	67	9.5	68
CNI trough 12 week after initiation (ng/ml)	92	79	66	9.0	65
ISDR mutation (number)	Mutant (9)	Wild (0)	Wild (0)	Intermediate (1)	Undeterminable
Core 70	Undeterminable	Wild	Mutant	Wild	Wild
Core 91	Undeterminable	Wild	Mutant	Wild	Wild
IL28B Recipient /Donor[b]	TT/TT	TG/TT	TG/TT	GG/TG	TT/TT

CNI calcineurin inhibitor, *CsA* cyclosporine A, *FK* tacrolimus, *GFR* glomerular filtration rate, *HCV* hepatitis C virus, *IFN* interferon, *MMF* mycophenolate mofetil, *RBV* ribavirin, *LT* liver transplantation

[a] As per Metavir

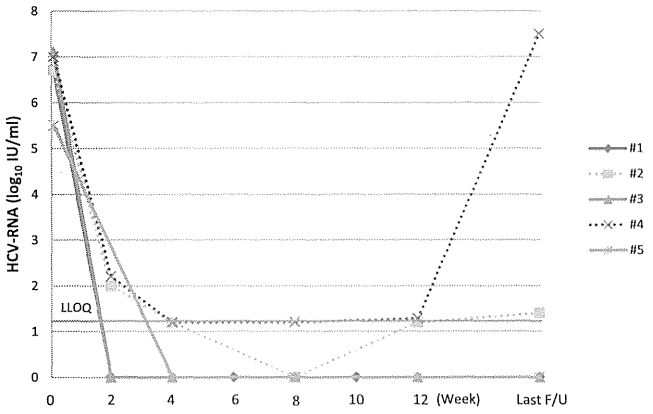
[b] Genotype rs8099917

trough level only moved from 9.8 to 9.0 ng/ml following the initiation of SMV.

After the completion of SMV, the CNIs were not restored to the original dose automatically, but modified according to the trough levels. Those without dose adjustment during the triple therapy (patient #3 and 5), the trough level at the last follow up were stable (67 and 61 ng/ml, respectively) with the same dose of CsA. Patient #2 showed lower trough level at week 20, and the dose of the CsA was re-increased to the original dose (75 to 100 ng/ml). The CNI dose of the remaining two patients (patient #1 and 4) were not changed since the completion of SMV to the last follow up with stable trough levels. The dose/use of MMF was not changed during the triple therapy throughout the follow up period, other than patient #1 who experienced diarrhea as noted above. There were no episodes of acute cellular or chronic (ductopenic) rejection observed during the study period.

Discussion

Here we present the results of a pilot study to reveal the characteristics of SMV-based triple anti-HCV treatment for



	w0	w4	w8	w12	Last F/U
#1	6.7	Undetectable	Undetectable	Undetectable	(w27) Undetectable
#2	6.7	<1.2	Undetectable	<1.2	(w23) 1.4
#3	7.1	Undetectable	Undetectable	Undetectable	(w22) Undetectable
#4	6.7	<1.2	<1.2	1.2	(w21) 7.5
#5	5.5	Undetectable	Undetectable	Undetectable	(w16) Undetectable

Fig. 2 Hepatitis C virus (HCV) RNA levels in five patients with simeprevir-based triple antiviral treatment. Each solid line represents an individual patient with an on-treatment virological response. Each dashed line represents an individual patient who did not achieve undetectable HCV RNA at week 12. The lower level of quantification (LLOQ) was 1.2 log10 IU/ml

LDLT recipients with recurrent hepatitis C. SMV became available after the introduction of TVR, which we have used in a selected patient group before the SMVs were introduced, and BOC into the liver transplant setting, thus a primary aim of the present study was to provide a preliminary report of the clinical experience with SMV in the liver transplant setting. Compared with TVR and BOC, the result of the current study suggested that the treatment with SMV was acceptably effective, with a rapid virologic response in three out of all five patients. In addition, importantly, no fatal adverse events, such as rejection, renal impairment, or severe cytopenia were observed.

We treated patients with SMV-based triple therapy as part of the pre-emptive therapy for recurrent hepatitis C. The rationale for this pre-emptive therapy is to strike at a time when histologic damage is minimal regardless of the clinical symptoms of recurrent HCV following transplantation [11, 19, 20]; thus we initiated SMV for those with even minimal or no graft injury due to recurrent hepatitis C, as long as the HCV remains persistent with dual treatment.

We investigated HCV polymorphisms at the NS3 position in all patients before the introduction of SMV. At baseline, none of the patients had mutations reported to reduce the antiviral effects of SMV *in vitro* [21]. Patient #2 had Q80L and V170I mutations at baseline; she achieved an undetectable HCV titer at week 8, whereas the other three patients achieved an undetectable HCV titer within the first 4 weeks, including two patients who relapsed with TPV-based triple therapy prior to the current study. The HCV-RNA of patient #2 became positive again at week 12, although it was around the LLOQ and not regarded as a breakthrough.

We also checked baseline polymorphisms at the NS5A position at the same time in anticipation of the coming treatment option with Daclatasvir (first-in-class, NS5A replication complex inhibitor) combined with Asunaprevir (NS3 protease inhibitor), which has been well tested in phase 3 clinical trial in Japan [22]. Patient #1, 3 and 4 had the Q54H mutation in NS5A, which might be associated with low-level resistance to an NS5A replication complex inhibitor [23]. Two out of those three patients achieved early virologic response. It seems feasible to introduce SMV-based triple therapy for such patients especially with some doubts about the potential efficacy of dual therapy with Daclatasvir and Asunaprevir in the liver transplant setting.

Importantly, there were no treatment cessations due to side-effects. One patient experienced grade 2 diarrhea, but this was resolved soon after the reduction of MMF: thus, it is difficult to determine whether SMV was the risk factor for diarrhea. Otherwise, no significant adverse events were observed, including elevation of serum total bilirubin. Necessary modifications in immunosuppression, especially CNIs, were also minimal. Technically it was not difficult for us to safely modify the dose of CNIs without a dose adjustment prior to the introduction of SMV, and comparatively mild modifications (50% to none) were required during the triple therapy. None of the five patients experienced renal dysfunction, infection, or rejection due to the uncontrolled trough level of CNIs, as noted above.

The introduction of TVR and BOC was anticipated to greatly improve virologic effects, even in liver transplant recipients with recurrent hepatitis C. The efficacy of TVR- or BOC-based triple therapy, however, was somewhat unsatisfactory; approximately 50% of the patients receiving such treatment achieved SVR [9, 24–27]. TVR- or BOC-based triple therapy was also associated with challenges in controlling the CNI trough levels and unignorable adverse events, such as cytopenic events, renal impairment, or skin rash [9]. In contrast, the previously reported profile of SMV is promising for liver transplant recipients with recurrent hepatitis C for the following reasons: first, the virologic effect is much greater than that of only Peg-IFN and RBV, with few side-effects by SMV itself [8, 28, 29], and second, SMV has few drug interactions with CNIs [10]. As demonstrated in the present study, the reported advantages of SMV in addition to TVR or BOC seem to be applicable to the management of post-transplant recurrent hepatitis C, with its safety and feasible virologic effect compared to TPV and BOC.

The present study has several limitations. The number of patients included was limited to only five, and all five patients were selected from among those receiving

pre-emptive antiviral therapy following liver transplantation with a poor virologic response. In addition, the five patients showed minimal or no graft damage when SMV was started. Hence, this study does not allow us to draw a robust conclusion regarding the use of SMV for liver transplant recipients, especially in evaluating the potential efficacy of SMV as a first-line treatment for recurrent hepatitis C. In addition, patients were followed only during the SMV-based triple therapy, and the actual virologic response after completing the treatment (i.e., 36 more weeks of dual therapy with Peg-IFN and RBV) should be evaluated. Further studies are warranted to address those concerns.

In conclusion, the present pilot study revealed the feasibility and safety of SMV in combination with Peg-IFN and RBV in LDLT recipients with recurrent hepatitis C. This combination therapy produced fewer side-effects and drug interactions with CNIs than prior DAAs. Recipients who were tolerant to dual therapy (Peg-IFN with RBV) but could not achieve a satisfactory viral response should be considered candidates for SMV. The actual profile of the current SMV-based antiviral treatment for recurrent hepatitis C post-liver transplantation, however, should be evaluated after the completion of a full course of therapy followed by 36 weeks of dual therapy with Peg-IFN plus RBV. In addition, future studies including a larger number of liver transplant recipients in diverse situations, such as those undergoing first-line treatment for established recurrence of HCV post-liver transplantation, are crucial.

Conflict of interest None declared.

Author contribution Study design: Tomohiro Tanaka, Yasuhiko Sugawara and Norihiro Kokudo. Acquisition of data: Nobuhisa Akamatsu, Junichi Kaneko, Sumihito Tamura, Taku Aoki, Yoshihiro Sakamoto, Kiyoshi Hasegawa. Analysis and interpretation: Tomohiro Tanaka, Masayuki Kurosaki, Namiki Izumi and Yasuhiko Sugawara. Manuscript drafted by: Tomohiro Tanaka, Nobuhisa Akamatsu, Masayuki Kurosaki and Yasuhiko Sugawara. Study supervision: Norihiro Kokudo.

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ORIGINAL ARTICLE

Effects of a whey peptide-based enteral formula diet on liver dysfunction following living donor liver transplantation

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Abstract
Background and aims Whey protein, a protein complex derived from milk is well known as a functional food with a number of health benefits. MEIN® (Meiji Dairies Co., Tokyo Japan) is a functional liquid-type nutritional diet containing whey-hydrolyzed peptide. In this study, we examined the effects of MEIN® on postoperative liver dysfunction in patients who underwent living donor-related liver transplantation (LDLT).
Methods Sixteen adult patients transplanted between 2005 and 2011 at our institute were evaluated retrospectively. In MEIN group ($n = 8$), administration of MEIN® was started around 14 days after liver transplantation when serum liver enzymes were re-elevated, while MEIN® was not administered in the control group ($n = 8$) who did not have postoperative liver dysfunction.
Results In the preoperative clinical characteristics, the model for end-stage liver disease score in the MEIN group was significantly lower than that in the control group. The graft-to-recipient body weight ratio in the MEIN group was lower than that in the control group. Elevation of enzymes in the liver function tests such as alanine aminotransferase and total bilirubin, and C-reactive protein in the MEIN group had significantly improved, and became almost normal values which were the same as those in the control group.
Conclusion These findings suggest that administration of whey-hydrolyzed peptide attenuates the post-transplant liver dysfunction and may avoid an unnecessary liver biopsy.

Keywords Liver transplantation · Whey peptide · Acute cellular rejection · Enteral nutrition

Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
CRP	C-reactive protein
CT	Computed tomography
GRWR	Graft-to-recipient body weight ratio
HBV	Hepatitis B virus
HCV	Hepatitis C virus
LDLT	Living related donor liver transplantation
LPS	Lipopolysaccharide
MRCP	Magnetic resonance imaging
MELD	Model for end-stage liver disease
T-Bil	Total bilirubin

Introduction

After liver transplantation, the levels of liver enzymes, such as aspartate aminotransferase (AST), and alanine aminotransferase (ALT), are often elevated due to acute cellular rejection, the recurrence of virus hepatitis, portal vein thrombosis, hepatic artery thrombosis, hepatic vein obstruction, bile duct complications, drug-induced liver injury, and various types of infection [1, 2]. The presence of vessel thrombosis or obstruction and bile duct complications can be determined by imaging modalities, such as ultrasonography (US), dynamic computed tomography

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(CT) or magnetic resonance imaging (MRI). In the patients with rejection or recurrence of hepatitis, a liver biopsy may be required [3, 4]; however, there may be some serious risks associated with such biopsies, such as bleeding, bile leakage or other organ injury. When the etiology of the elevation of liver enzymes can be determined, the liver biopsy may be avoidable [5–8].

Careful perioperative management, including defined nutrition, should be considered for patients undergoing liver transplantation [9]. Several studies have shown that immune-modulating nutritional formulas may have a role in improving the preoperative nutritional status, hastening recovery after transplantation, and reducing postoperative infectious complications [10]. Therefore, we retrospectively evaluated the effects of immune-modulating formulas in recipients after living donor-related liver transplantation (LDLT). In this study, we used a whey-hydrolyzed peptide for the formula, which is a protein complex derived from milk. It has been reported to have antioxidant, antihypertensive, antitumor, antiviral, hypolipidemic, and antibacterial effects [11]. The whey proteins from milk include β-lactoglobulin, α-lactalbumin, glycomacropeptide, immunoglobulins, and lactoferrin, and are used as a functional food that is considered to provide a number of health benefits [11]. These proteins also have been reported to exert anti-inflammatory and hepatoprotective effects [12–15]. Whey-hydrolyzed peptide has hepatoprotective effects against hepatitis and is more easily absorbed than whey protein. A previous study showed that the serum lipid peroxide levels significantly decreased, and the interleukin (IL)-2 levels and natural killer (NK) activity significantly increased in patients with chronic hepatitis due to hepatitis B virus (HBV) and C virus (HCV) infection following consumption of whey-hydrolyzed peptide [16].

MEIN® (Meiji Dairies Co., Tokyo, Japan) contains an abundance of whey-hydrolyzed peptide, which is extracted from bovine milk. This nutritional formula, like other whey-derived proteins, has been reported to have antioxidant, antihypertensive, antitumor, antiviral, hypolipidemic, and antibacterial effects in vivo and in vitro [11, 14, 17–19]. Moreover, early enteral nutrition with MEIN® was useful to prevent post-LDLT bacteremia and shorten the postoperative hospital stay in transplant patients [20].

In the present study, we evaluated the usefulness of MEIN® including a whey-hydrolyzed peptide for patients with re-elevation of the liver enzyme levels after LDLT.

Patients and methods

Study design and enrolled patients

Eight adult patients who received transplants between 2005 and 2011 at Tokushima University Hospital were evaluated retrospectively. The indication for LDLT was HCC in three cases, HCV infection in three cases, HBV infection in one case and Wilson's disease in one case (Table 1). Eight patients who did not have postoperative liver dysfunction and did not receive the MEIN formula served as the control group.

Table 1 Patients characteristics

Background	MEIN ($n = 8$)	Control ($n = 8$)	p value
Age	49 ± 13	55 ± 3	0.21
Gender (F/M)	3/5	4/4	0.25
Indication for LDLT			
HCC	3	0	
HCV-related liver cirrhosis	3	1	
HBV-related liver cirrhosis	1	4	
Others	1	3	
Child-Pugh classification A/B or C	2/6	0/8	N.A
MELD score	10 ± 4	16 ± 6	0.04
ABO compatibility			
Identical/compatible	6	8	N.A
Incompatible	2	0	
Graft type (left lobe/right lobe)	7/1	6/2	0.41
Graft versus recipient weight (GRWR)	0.72 ± 0.12	0.89 ± 0.19	0.06

Perioperative management of LDLT

Liver transplantation was performed using a living related donor. The surgical procedures for the donor and recipient have been described previously [21]. For immunosuppressive therapy, induction consisted of two doses of basiliximab (Simulect®, NOVARTIS) on postoperative days 0 and 4. Standard immunosuppressive therapy at discharge consisted of corticosteroids and calcineurin inhibitors (either tacrolimus or cyclosporine) with mycophenolate mofetil (MMF). Prednisolone was discontinued on day 21 after the surgery. In ABO incompatible cases, we administered preoperative anti-CD20 antibodies (Rituximab®, 375 mg/m^2) and performed plasma exchange for 3 days.

MEIN® composition

A commercially available enteral nutrition, MEIN® (Meiji Dairies Corporation, Tokyo, Japan) was used in this study. It is a newly designed enteral formula, including whey peptide. In terms of its general composition, it has 1 kcal/ml, including 50 mg/ml of protein, 28 mg/ml of fat, 133 mg/ml of carbohydrate, 12 mg/ml of alimentary fiber, 6 mg/ml of

ash content, and is made using 84.4 g/100 ml of water. Moreover, it includes 2.25 g/100 ml of essential amino acids and 2.63 g/100 ml of nonessential amino acids. The Fischer ratio is 3.7. The protein sources used for MEIN® are whey-hydrolyzed peptide and fermented milk.

Administration of MEIN®

The administration of MEIN® was started 14.6 ± 2.4 days after liver transplantation in the patients ($n = 8$) who showed a re-elevation of liver enzyme levels (MEIN group). The patients were administered MEIN® three times a day either orally or through a tube jejunostomy (Fig. 1).

Blood biochemistry

All patients were monitored for the liver enzyme levels, including AST and ALT, alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γGTP), as well as the total bilirubin (T-Bil) and C-reactive protein (CRP) levels as parameters of liver dysfunction before the administration of MEIN, after 7 days of administration and 14 days after starting the administration of MEIN.

Statistical analysis

Statistical comparisons of the mean values were conducted using a one-way analysis of variance (ANOVA). All results are presented as the mean ± standard deviation (SD). A p value <0.05 was considered to be statistically significant. The statistical analysis was performed using the JMP® 7.0.2 statistical software program (SAS Institute, Cary, NC).

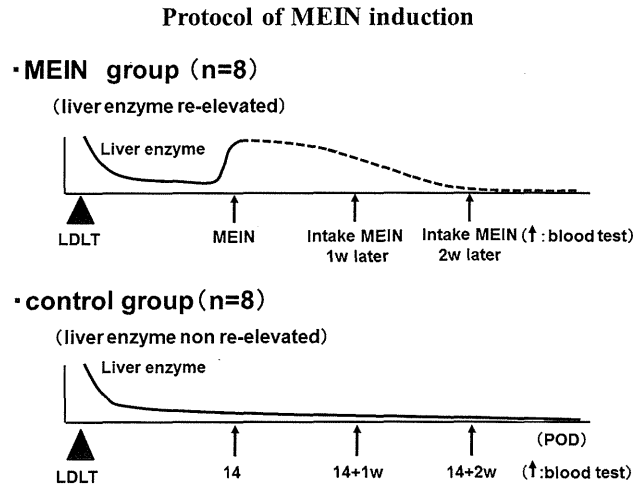

Fig. 1 The timing of the re-elevation of liver enzyme levels and the administration of MEIN

Results

Patient characteristics

The model for end-stage liver disease (MELD) score in the MEIN group was significantly lower than that in the control group (10 ± 4 vs. 16 ± 6, $p = 0.04$) (Table 1). In the control group, all of the patients categorized as having Child B/C status, while there were two Child A patients in the MEIN group. In the control group, there were no ABO incompatible cases, while there were two ABO incompatible cases in the MEIN group. The graft-to-recipient body weight ratio (GRWR) in the MEIN group was lower than that of the control group (0.72 ± 0.12 vs. 0.89 ± 0.19, $p = 0.06$). There were no significant differences in any of the other characteristics, including the patient age, gender or graft type.

Blood biochemistry

The serum levels of AST and ALT 1 and 2 weeks after starting the administration of MEIN® were significantly lower than those before MEIN® administration (AST: 101.4 ± 61.5 vs. 52.3 ± 31.4 vs. 45.8 ± 20.5, ALT: 201.1 ± 133.9 vs. 123.1 ± 104.2 vs. 79.9 ± 47.8, $p < 0.05$). The serum levels of T-Bil and CRP 2 weeks after starting the administration of MEIN® were significantly lower than those before MEIN® administration (T-Bil: 4.3 ± 4.9 vs. 2.5 ± 4.5, CRP: 1.7 ± 1.0 vs. 0.8 ± 0.7, $p < 0.05$) (Fig. 2a, b). After 2 weeks of MEIN, these values were almost identical to those values in the control group. The serum levels of ALP and γGTP did not differ significantly in the patients between before and after the administration of MEIN®.

Discussion

Patients often experience a re-elevation of liver enzyme levels around 2 weeks after LDLT, even after the early postoperative liver dysfunction is improved. In such cases, it is necessary to consider several possible etiologies, such as acute cellular rejection, recurrence of virus hepatitis, portal vein thrombosis, bile duct complication, and drug-induced liver injury, in order to optimize the treatment strategy. It is worth noting that the administration of an enteral formula (MEIN®), which contains whey-hydrolyzed peptide, significantly improved the re-elevated liver enzyme levels after LDLT in the present study. This is the first report demonstrating that whey-hydrolyzed peptide can ameliorate the liver dysfunction in patients after LDLT.

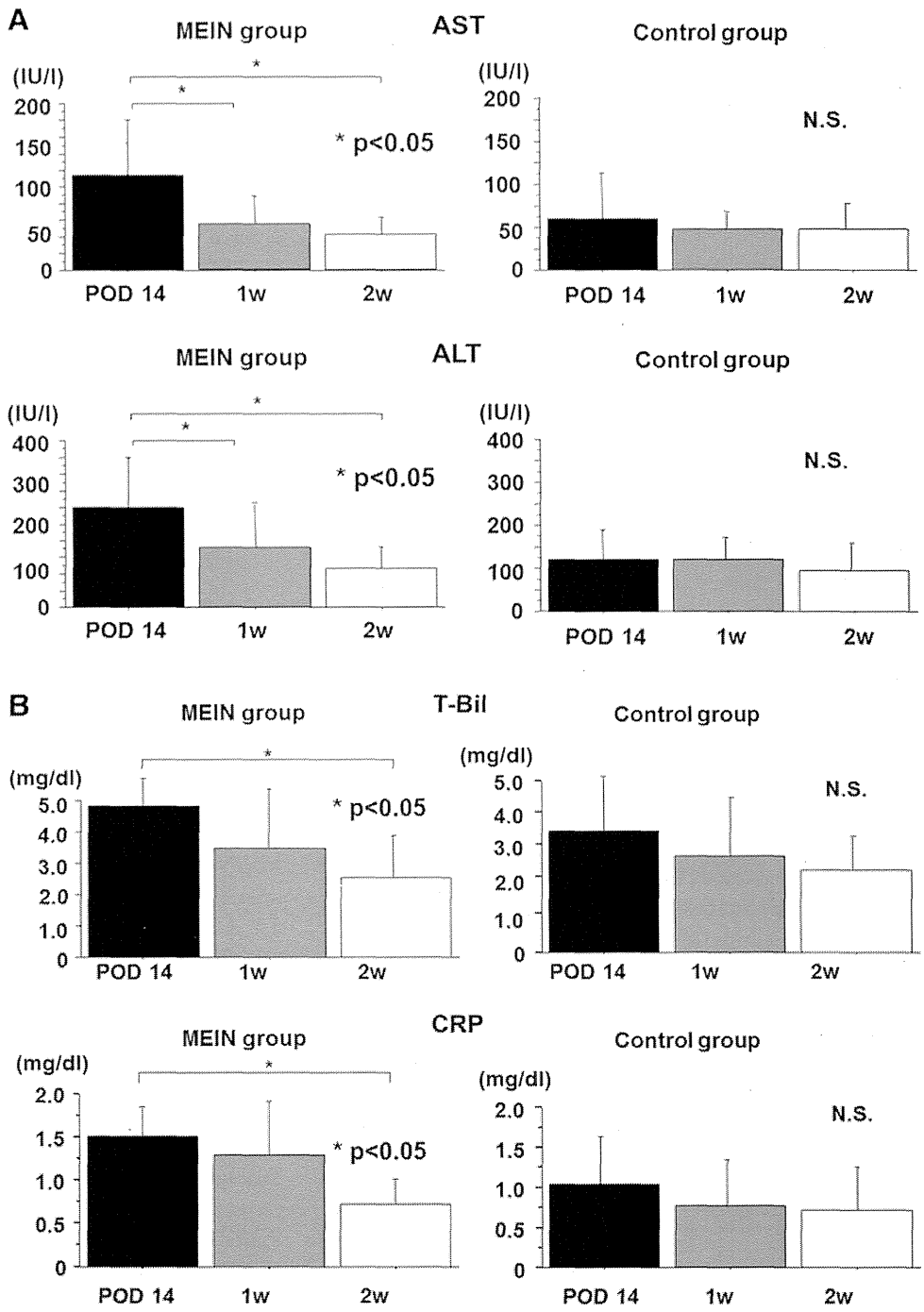

Fig. 2 The results of the biochemical analyses of the patients in the MEIN and control groups. **a** Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), **b** total bilirubin (T-Bil) and C-reactive protein (CRP) levels

Kume et al. [13] previously reported that whey-hydrolyzed protein has hepatoprotective effects against D-galactosamine-induced hepatitis and liver fibrosis in rats by suppressing IL-6. In the burn rat model, whey-hydrolyzed peptide led to a significant increase in hepatic glutathione levels 4 h after burn injury. The hepatic and renal lipid peroxide levels were increased 4 h after burn injury in the rats fed a standard diet. Whey supplementation significantly suppressed the burn-induced increase in the hepatic and renal lipid peroxide levels. Whey-hydrolyzed peptide also suppressed the hepatic and renal oxidative stress after experimental burn injury [14]. Recently, it was reported that MEIN® demonstrated anti-inflammatory effects and protected against concanavalin-A induced hepatitis in mice by suppressing the production of inflammatory cytokines [22].

The mucosal secretion of lactoferrin, which is composed of whey-hydrolyzed peptide, a glycoprotein present in milk, contributes to the host defense. Harversen et al. [15] have previously shown that orally given milk lactoferrin

mediates anti-infectious and anti-inflammatory activities in vivo. They also showed that lactoferrin could down-regulate the lipopolysaccharide (LPS)-induced IL-6 secretion in a human monocytic cell line. Moreover, Hara et al. [12] reported that lactoferrin can also inhibit HCV and HBV infections in cultured human hepatocytes. Pre-incubation of the cells with bovine or human lactoferrin prevented the HBV infection of the cells. This report suggested that the interaction of lactoferrin with cells was important for its inhibitory effect, and that lactoferrin may be a candidate anti-HBV agent that could prove to be effective for the treatment of patients with chronic viral hepatitis.

In a recent clinical prospective study involving thirty adult patients, MEIN® was administered to ten patients who underwent LDLT and twenty patients (as controls) received a conventional enteral diet as the formula for early enteral nutrition. The incidence of bacteremia was significantly lower in the MEIN group than the control group (10 vs. 50 %, $p = 0.032$). The mean length of postoperative hospital stay after LDLT was significantly shorter in the MEIN group than that in the control group (45 ± 12 vs. 71 ± 34, $p = 0.018$) [23]. In a more recent study, it was shown that early administration of MEIN® could prevent post-transplant bacteremia in 76 consecutive patients [24].

Based on these previous studies and our current findings, we propose a flow chart for the management of patients with re-elevation of serum liver enzymes after LDLT, as shown in Fig. 3. If the patient shows re-elevation, diagnostic imaging, including US, CT or MRCP and blood tests should be performed to exclude blood flow disturbances, such as thrombosis or stenosis, bile duct complications or a recurrence of hepatitis virus infection. If the cause of the re-elevation is determined to be one of these etiologies, adequate management for such an etiology should be conducted. On the other hand, if the cause of the re-elevation cannot be clearly identified, then MEIN® should be administered. If the levels do not recover, a liver biopsy may be performed to rule out other etiologies, such as acute cellular rejection. However, since the number of patients included in this retrospective study was small, this flow chart should be confirmed in a prospective study involving a larger number of LDLT patients.

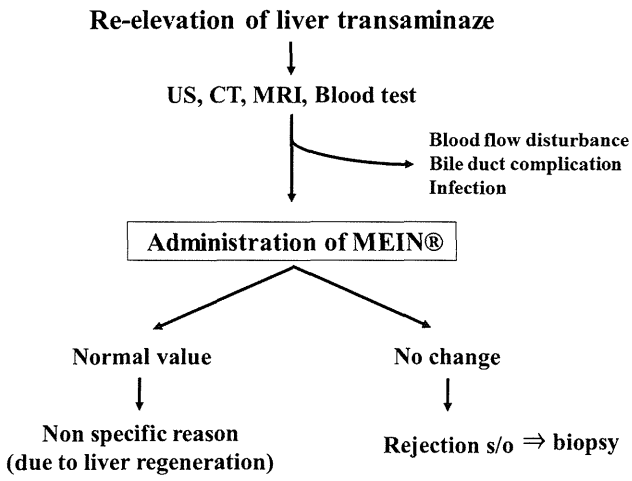

Fig. 3 A proposed flow chart of the postoperative management of patients who show a re-elevation of AST and ALT after LDLT

In conclusion, the administration of MEIN® can attenuate the re-elevation of liver enzyme levels after LDLT, and may help avoid the need for a liver biopsy.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Beneficial effects of green tea catechin on massive hepatectomy model in rats

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Abstract
Background Green tea catechin, especially epigallocatechin gallate (EGCG), is a well-known scavenger of reactive oxygen species and it may also function as an antioxidant through modulation of transcriptional factors and enzyme activities.
Methods Green tea extract (GTE®) which contained numerous EGCG was used. Wistar rats were performed 90 % hepatectomy and classified into 2 groups with (GTEHx, $n = 25$) or without GTE treatment (Hx, $n = 25$) and sacrificed at 1, 3, 7 and 14 days after operations. All rats had free access to drinking water supplemented with or without GTE from the 7th pre-operative day. Liver regeneration, hepatic inducible nitric oxide synthase (iNOS), anti-oxidative enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px)] and inflammatory markers [cyclooxygenase-2 (COX-2), nuclear factor kappa B (NFκB), tumor necrosis factor-α (TNF-α)] were investigated.
Results The liver weight to body weight ratio ($p < 0.01$), proliferating cell nuclear antigen labeling index ($p < 0.05$) and phosphorylated extracellular signal-regulated kinase 1/2 ($p < 0.05$) at day 1 in the GTEHx group significantly increased compared to the Hx group. Hepatic iNOS levels at day 1 significantly decreased ($p < 0.01$) in the GTEHx group. Hepatic SOD, CAT and GSH-Px levels at day 1 significantly increased (SOD: $p < 0.01$, CAT and GSH-Px: $p < 0.05$) in the GTEHx group. In contrast, COX-2, NFκB and TNF-α levels at day 1 significantly decreased (COX-2: $p < 0.01$, NFκB and TNF-α: $p < 0.05$) in the GTEHx group.
Conclusions GTE pretreatment stimulated liver regeneration and improved liver damage after massive hepatectomy through anti-oxidative and anti-inflammatory effects. Green tea catechin might have the potential to attenuate liver dysfunction in early stage after massive hepatectomy.

Keywords Green tea catechin · Anti-oxidative · Anti-inflammatory · Massive hepatectomy

Abbreviations

ROS	Reactive oxygen species
iNOS	Inducible nitric oxide synthase
EGCG	Epigallocatechin gallate
EC	Epicatechin
EGC	Epigallocatechin
ECG	Epicatechin gallate
GTE	Green tea extract
MAPK	Mitogen-activated protein kinase
ERK ½	Extracellular signal-regulated kinase 1/2
KCs	Kupffer cells
MDA	Malondialdehyde
SOD	Superoxide dismutase
CAT	Catalase

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GSH-Px	Glutathione peroxidase
COX-2	Cyclooxygenase-2
NFκB	Nuclear factor-kappa B
TNF-α	Tumor necrosis factor-α
Lw/Bw	Liver weight to body weight
PCNA LI	Proliferating cell nuclear antigen labeling index
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
LDH	Lactase dehydrogenase
T-Bil	Total bilirubin

Introduction

Although massive hepatectomy is sometimes required to achieve a curative resection for advanced hepatic malignancies, most instances of mortality after such surgery are still attributed to hepatic failure with small remnant liver volume [1]. Therefore, innovative strategies are required for the treatment of hepatic insufficiency after massive hepatectomy. Previously, we reported that hyperbaric oxygen pretreatment had beneficial effects in a massive hepatectomy model in rats via the induction of heat shock protein 70 and hemeoxygenase 1 [2]. In addition, we have also shown the beneficial effects of fluvastatin in lethal massive hepatectomy model rats with improved hepatic regeneration and microcirculations by inhibiting the activation of hepatic stellate cells [3].

Various hypotheses have been reported for the mechanisms of hepatic failure after massive hepatectomy. Pro-inflammatory mediators and reactive oxygen/nitrogen species (ROS/RNS) are excessively produced in the liver after major hepatectomy [4, 5]. The up-regulation of inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β), and others [6, 7] plays an important role in the pathogenesis of hepatic injury through the induction of neutrophil adhesion to the endothelial cells [8] and microcirculatory disturbance [9]. The production of superoxide and other ROS, derived from the activation of various enzymes (e.g., xanthine oxidoreductase) [10, 11] also plays a critical role in tissue damage. Additionally, the presence of excessive amounts of nitric oxide (NO), mostly produced by the up-regulation of inducible nitric oxide synthase (iNOS) in response to hepatic stress, combines with ROS to favor the formation of the potent oxidant peroxynitrite ($ONOO^-$) [12] and disrupting cellular functions [13]. Elevated levels of both ROS/RNS and inflammatory cytokines such as IL-6 and TNF-α also activate various cell death signaling pathways [7, 14–16] leading to apoptosis and/or necrosis.

Green tea catechin, especially epigallocatechin gallate (EGCG), is a well-known scavenger of ROS [17, 18], and it may also function as an antioxidant through modulation of transcriptional factors and enzyme activities [19]. Recently, a few reports have been published regarding the beneficial effects of EGCG for liver fibrosis. Yasuda et al. [20] reported that EGCG prevented carbon tetrachloride (CCl_4)-induced rat hepatic fibrosis by inhibiting the expression of the platelet-derived growth factor receptor β (PDGFRβ) and insulin-like growth factor-1 receptor (IGF-1R). Tipoe et al. [21] also reported that EGCG significantly attenuated the severity of CCl_4-induced liver injury and the progression of liver fibrosis. The protective effect of EGCG may be a consequence of the reduction in oxidative stress and the pro-inflammatory response.

Therefore, it is possible that green tea catechin, being rich in EGCG, may be a dietary antioxidant which can be used clinically in liver dysfunction after massive hepatectomy. In this study, we demonstrated that preoperative administration of green tea catechin stimulated liver regeneration and improved liver damage after massive hepatectomy in rats.

Methods

Animals

6-week-old male Wister rats, weighing 180–220 g, were obtained from Charles River Laboratories (Kanagawa, Japan). Animals were provided with water and standard laboratory diet for at least 7 days before use. Throughout the experiment, the animals were maintained behind barriers under controlled conditions and had free access to tap water and food before and after operations. The present study was conducted in compliance with the Division for Animal Research Resources, Institute of Health Biosciences, University of Tokushima. The experiment and procedures were approved by the Animal Care and Use Committee of the University of Tokushima.

Administration of green tea catechin

Green tea extract (GTE®) utilized in powder form was purchased from the Green Tea Union of Saitama (Iruma Kumiai Seicha), Saitama, Japan. 500 mg GTE powder contains 52.5 mg EGCG, 12.3 mg epicatechin (EC), 34.6 mg epigallocatechin (EGC), 11.1 mg epicatechin gallate (ECG), and 15.7 mg caffeine (Table 1). 500 mg GTE powder is approximately equivalent to 2 Japanese-size cups of green tea. GTE powder was dissolved in sterilized water and administered to rats for 7 days preoperatively with free access to drinking tap water [20, 22].

Table 1 Constitution of GTE®

Constitution	Contained amount
Epigallocatechin gallate (EGCG)	52.5
Epicatechin (EC)	12.3
Epigallocatechin (EGC)	34.6
Epicatechin gallate (ECG)	11.1
Caffeine	15.7

mg/500 mg GTE®

Dissolved concentration of GTE was decided to 0.5 % for fear of rats' weight loss.

Surgical procedures

Operations were performed under light isoflurane anesthesia. 90 % hepatectomy was performed modifying the technique of Higgins and Anderson [23]. Animals were sacrificed at 1, 3, 7 and 14 days after hepatectomy. Immediately before sacrifice, blood samples were obtained from the superior vena cava for biochemical analysis. The livers were harvested by midline laparotomy, followed by dissection under light isoflurane anesthesia. Whole livers were removed, weighed, and one part of the caudate lobes was put in RNA later and stored at −80 °C until RNA extraction for the real-time RT-PCR, and another part was stored at −80 °C until use for western blots, and the other part was fixed in 10 % formaldehyde for an immunohistochemistry.

Experimental protocol

Rats were randomly divided into the following 2 groups. Group 1: simple laparotomy and 90 % hepatectomy with sterilized water administered for 7 days preoperatively (Hx, $n = 25$). Group 2: 90 % hepatectomy after administration of sterilized water supplemented with GTE and administered for 7 days preoperatively (GTEHx, $n = 25$) (Fig. 1). All rats had free access to drinking water (tap water supplemented with or without GTE). As previously described, dissolved concentration of GTE was decided to 0.5 %. In this study, on average, all rats drank almost 500 ml water supplemented with or without GTE per a week. Then the degree of liver regeneration, hepatic mitogen-activated protein kinase (MAPK) such as extracellular signal-regulated kinase 1/2 (ERK 1/2) and the status of kupffer cells (KCs) in regenerative livers were investigated. Serum liver function tests were also performed to evaluate the liver damage. We subsequently evaluated serum malondialdehyde (MDA) as an oxidative marker using the ELISA method and hepatic iNOS, anti-oxidative enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px)] and inflammatory markers

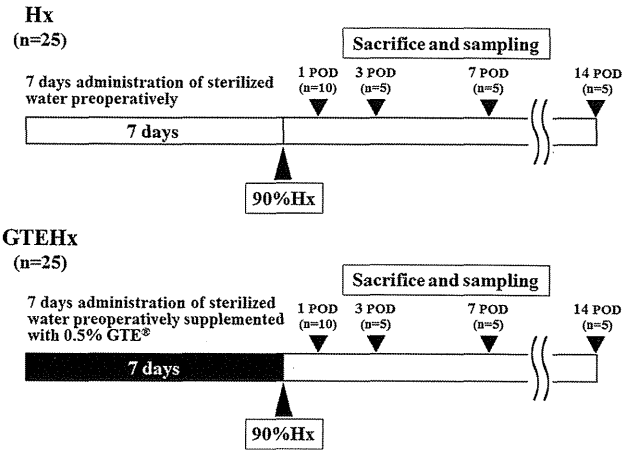

Fig. 1 Study design. Rats were divided into the 2 groups. In the Hx group ($n = 25$), we performed simple laparotomy and 90 % hepatectomy for rats with sterilized water administered for 7 days preoperatively. In the GTEHx group ($n = 25$),we performed 90 % hepatectomy as well for rats after administration of sterilized water supplemented with 0.5 % GTE and administered for 7 days preoperatively. In both groups, animals were sacrificed at 1 ($n = 10$), 3 ($n = 5$), 7 ($n = 5$) and 14 ($n = 5$) days after operations

[cyclooxygenase-2 (COX-2), nuclear factor kappa B (NFκB), TNF-α] using real time RT-PCR.

Immunohistochemistry for PCNA and estimation of liver regeneration

Excised liver specimens were fixed in 10 % formaldehyde and embedded in paraffin. Immunohistochemical staining was performed on 4 μm sections using the anti-rat proliferating cell nuclear antigen (PCNA) antibody (1:2,000, sc-56, Santa Cruz, CA, USA). Overnight incubation at 4 °C with primary antibody and indirect immunoperoxidase staining with the avidin–biotin complex (Dako, Glostrup, Denmark) and DAB Tablet (Wako Pure Chemical Industries Ltd., Osaka, Japan) were applied for visualization of the antigens. Hepatocytes with brown nuclei were considered to show PCNA-positive staining [24]. Sections were examined at a magnification of 400×, and 5 fields were randomly chosen to determine the PCNA labeling index (LI). The LIs were determined from more than 1,000 nuclei and were expressed as the percentage of hepatocytes showing positive staining.

Liver regeneration was defined as liver weight to body weight (Lw/Bw) and PCNA LI which was already established in previous in vivo studies [2, 3].

Western blots analysis of total and phosphorylated ERK 1/2

Liver samples with equal amounts of protein were homogenized in liquid nitrogen and lysed in sodium