Usefulness of Transient Elastography for Noninvasive and Reliable Estimation of Right-Sided Filling Pressure in Heart Failure

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Accurate noninvasive assessment of right atrial pressure (RAP) is important for volume management in patients with heart failure (HF). Transient elastography is a noninvasive and reliable method to assess liver stiffness (LS). We investigated the value of LS for evaluation of RAP in patients with HF without structural liver disease. We measured LS using transient elastography (Fibroscan) in 31 patients undergoing right-sided cardiac catheterization (test group). The relation between LS and RAP found in the test group was used to derive the best-fit model to predict RAP. The applicability of the model was then tested in a validation group of 49 additional patients. There was an excellent correlation between LS and RAP in the test group ($r = 0.95$, $p < 0.0001$; $RAP = -5.8 + 6.7 \times \ln[LS]$). Natural log transformation (ln) of LS provided the regression equation to predict RAP. When the equation model derived from the test group was applied to the validation group, predicted RAP correlated excellently with actual RAP ($r = 0.90$, $p < 0.0001$). The receiver operating characteristic curve analyses in the test group showed that LS favorably compared with echocardiography for detecting RAP >10 mm Hg (area under the curve 0.958 vs 0.800, respectively, $p = 0.047$). In the validation group, LS with a cut-off value of 10.6 kPa for identifying RAP >10 mm Hg had a higher sensitivity and accuracy ($p = 0.046$ and $p = 0.049$, respectively) than echocardiography. In conclusion, LS may offer an accurate noninvasive diagnostic method to assess RAP in patients with HF. **(Am J Cardiol 2014;113:552–558)**

Increased right atrial pressure (RAP) causes liver congestion in most patients hospitalized with acute heart failure syndrome.[1] Anatomically, the liver is wrapped in a distensible but nonelastic envelope. Thus, liver congestion due to RAP elevation may lead to increased liver stiffness (LS). One experimental study of Landrace pigs showed that LS derived from transient elastography is directly influenced by central venous pressure in a reversible manner.[2] Transient elastography is a noninvasive and reliable approach that has been introduced to assess LS by measuring the liver shear wave speed; however, the method has mainly been studied to stage liver fibrosis.[3] In the present study, we hypothesized that in the absence of structural liver disease, LS as assessed by transient elastography can be used to evaluate RAP in patients with heart failure (HF). To test this hypothesis, we investigated the relation between LS and invasively measured RAP. Furthermore, we compared the predictive value of LS with that of conventional echocardiography for identifying elevated RAP.

Methods

Patients admitted with HF and scheduled for right-sided cardiac catheterization were prospectively enrolled over a period of 8 months. Right-sided cardiac catheterization was indicated in patients who required accurate hemodynamic monitoring because of clinically indeterminate volume and those refractory to initial therapy. Patients being considered for cardiac transplantation or placement of a mechanical circulatory support system were also included. The diagnosis of HF was primarily based on signs and symptoms derived from a thorough history and physical examination.[4] Patients with history or signs of liver disease on hepatic ultrasound or invalid LS measurements due to severe obesity, narrow intercostal space, and substantial ascites were excluded from the study. The study protocol was approved by the institutional ethical committee, and written informed consent was obtained from all patients.

Both LS monitoring and echocardiographic assessments were performed within 3 hours before right-sided cardiac catheterization by a different examiner for each examination. The examiners were blinded to each other's results. Routine laboratory testing was performed on the same day. To identify the best model for deriving RAP from LS, the patients were divided into 2 subgroups: the test group and

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the validation group. We first obtained a regression equation between RAP and LS in the initial population (test group) and subsequently tested it in the second population (validation group). Finally, we developed the revised equation model for estimating RAPs from LS values in all patients. Thereafter, we compared the performance of LS for predicting RAP elevation with the conventional echocardiographic approach. LS was measured at the end-expiratory period using transient elastography with a Fibroscan device (Echosens, Paris, France).[5] The tip of the transducer was placed vertically on the skin between the rib bones at the level of the right liver lobe while the patient was lying supine with the right arm fully abducted. All measurements were done by a single certified examiner (TT) who had previously performed >500 determinations. Absolute and median values are expressed in kilopascals (kPa). When LS values showed an interquartile range/median ratio >0.25 or a success rate <60%, they were considered invalid. In each patient, up to 10 successful measurements were performed, and individuals with invalid measurements were excluded from the final analysis. LS examination was performed in <5 minutes in all cases.

Right-sided cardiac catheterization was performed in the cardiac catheterization laboratory or cardiac care unit using a flow-directed pulmonary artery catheter. Pressure calibration was performed before and after pressure measurements. All readings were referenced to the midaxillary line with the patient in the supine position. Pressure measurements were determined at the end-expiratory period, with an average of 3 to 5 cycles obtained. Cardiac output was measured by the assumed Fick equation.

Echocardiography was performed by an experienced operator using a Vivid q portable ultrasound system equipped with a 1.5- to 3.6-MHz 3S-RS probe (GE, Healthcare, Milwaukee, Wisconsin), as previously described.[6] Routine echocardiographic measurements were obtained according to the guidelines of the American Society of Echocardiography.[7] The maximal and minimal inferior vena cava (IVC) diameters were measured within 3 cm of the IVC-right atrium junction during passive respiration.[8] Echocardiographically estimated RAP was measured semiquantitatively based on IVC size and respiratory collapse rate according to American Society of Echocardiography criteria.[8]

Intra- and interobserver agreements were analyzed by calculating the intraclass correlation coefficient[9] in 10 randomly selected patients. Intrarater agreement was calculated as the agreement between the first and second LS of the single observer (TT), whereas interrater agreement was calculated as the agreement between the first LS of the 2 observers (TT and YT).

Statistical analyses were performed using JMP software, version 10.0 (SAS Institute, Cary, North Carolina) and SPSS for Windows, version 20.0 (SPSS Inc., Chicago, Illinois). A 2-sided p value <0.05 was considered statistically significant. Bland-Altman plots were used to show agreement between invasively determined RAP and LS-derived RAP, by plotting the difference against the mean.[10] Categorical data are presented as percentages, normally distributed continuous data are presented as mean ± SD, and nonnormally distributed variables are presented as median and interquartile range. Kruskal-Wallis tests were used to compare continuous variables between groups, and chi-squared tests were used for categorical variables. For each continuous variable, linearity was assessed and an appropriate transformation applied, if necessary. Natural log transformation (ln) of LS was used to satisfy the model assumptions; the "best-fit" line of the relation between LS and RAP was determined by simple linear regression analysis. Comparison between regression lines was made by analysis of covariance. Receiver operating characteristic (ROC) curves and area under the curve were computed to (1) compare the effectiveness of transient elastography and echocardiography and (2) examine their optimal sensitivity and specificity to predict RAP elevation. An RAP of >10 mm Hg was considered a clinically significant elevation in RAP.[11] For comparison of correlated ROC areas, the method described by DeLong et al[12] was used. Relations between LS and other variables were assessed by Pearson, Spearman, and point-biserial correlation coefficients. Only variables with a probability value <0.1 in univariate analysis were entered into multivariate analysis. Stepwise multiple linear regression analyses were performed to identify the determinants of LS.

Table 1
Indications for right-sided cardiac catheterization and clinical diagnosis

Clinical Diagnosis	Value, n = 80
Dilated cardiomyopathy	21 (26)
After valvular surgery*	12 (15)
Valvular heart disease†	5 (6)
After heart transplantation	5 (6)
Pulmonary hypertension	5 (6)
After coronary artery bypass surgery	4 (5)
Cardiac sarcoidosis	4 (5)
Ischemic cardiomyopathy	3 (4)
Pulmonary embolization	3 (4)
Hypertrophic cardiomyopathy	3 (4)
HF with preserved ejection fraction	3 (4)
Constrictive pericarditis	3 (4)
Acute coronary syndrome	2 (3)
Atrial septal defect	2 (3)
Fulminant myocarditis	2 (3)
Drug-induced cardiomyopathy	1 (1)
Sick sinus syndrome	1 (1)
Ventricular tachycardia	1 (1)

Values are expressed as number (percentage).

* Of "after valvular surgery," 3 were aortic and mitral valve replacements, 3 were isolated mitral valve replacement, 3 were isolated aortic valve replacement, 2 were isolated mitral valve repair, and 1 was isolated pulmonary valve replacement.

† Of valvular heart disease, 4 were severe aortic stenosis and 1 was severe mitral regurgitation.

Results

In total, 105 subjects were screened for this study. Sixteen patients were excluded before scanning because of organic hepatic diseases. Of 89 consecutive patients enrolled in the study, 9 patients with invalid LS were also excluded: 5 patients with interquartile range >0.25 and 4 with success rate <60%. Indications for right-sided cardiac catheterization and clinical diagnosis are presented in Table 1. In the remaining 80 patients, initial 31 patients were analyzed for

Table 2
Characteristics of the test and validation groups

Variable	Test Group (n = 31)	Validation Group (n = 49)
Age (yrs)	57 ± 17	58 ± 18
Men	22 (78%)	38 (78%)
Body mass index (kg/m^2)	22.1 (18.7–24.4)	21.9 (19.4–25.3)
Body surface area (m^2)	1.66 ± 0.18	1.67 ± 0.20
New York Heart Association functional class, I/II/III/IV	3/10/10/8	3/25/9/12
On mechanical ventilation	6 (20%)	5 (10%)
LS (kPa)	8.5 (5.3–12)	9.5 (5.9–15.3)
Hemoglobin (g/dL)	12.0 (9.6–13.5)	12.1 (9.8–14.1)
Platelet count (10^4/mm^3)	18.4 ± 8.4	17.9 ± 5.9
Aspartate aminotransferase (IU/L)	24 (18–42)	25 (19–45)
Alanine aminotransferase (IU/L)	20 (12–31)	25 (15–35)
γ-Glutamyl transpeptidase (IU/L)	58 (31–91)	64 (38–124)
Alkaline phosphatase (IU/L)	230 (161–331)	236 (186–331)
Lactic dehydrogenase (IU/L)	222 (183–368)	219 (191–312)
Total bilirubin (mg/dl)	0.7 (0.5–1.1)	0.8 (0.5–1.5)
Total cholesterol (mg/dl)	168 ± 44	166 ± 38
Triglycerides (mg/dl)	94 (59–165)	112 (66–164)
High-density lipoprotein cholesterol (mg/dl)	48 ± 18	47 ± 14
Low-density lipoprotein cholesterol (mg/dl)	95 ± 34	92 ± 27
Albumin (g/dl)	3.8 (3.3–4.2)	3.9 (3.3–4.3)
Estimated glomerular filtration ratio (ml/min/1.73 m^2)	61 ± 28	51 ± 24
B-type natriuretic peptide (pg/ml)	274 (69–610)	275 (57–534)
Mean RAP (mm Hg)	9 (5–12)	9 (6–14)
Heart rate (beats/min)	75 (67–93)	78 (66–89)
Systolic blood pressure (mm Hg)	106 (90–114)	102 (91–121)
Diastolic blood pressure (mm Hg)	59 (56–64)	58 (52–65)
Systolic pulmonary artery pressure (mm Hg)	35 (28–48)	36 (27–46)
Diastolic pulmonary artery pressure (mm Hg)	17 (12–25)	18 (13–22)
Pulmonary capillary wedge pressure (mm Hg)	14 (10–25)	17 (11–23)
Cardiac output (L/min)	3.63 (3.27–4.82)	3.67 (2.75–4.58)
Cardiac index (L/min/m^2)	2.26 (1.80–2.84)	2.10 (1.76–2.70)
Maximal IVC diameter (mm)	18 (11–22)	17 (13–23)
Minimal IVC diameter (mm)	15 (5–19)	11 (5–17)
Estimated RAP (mm Hg)	8 (3–15)	8 (3–15)
Left ventricular end-diastolic dimension (mm)	51 (46–70)	54 (48–67)
Left ventricular end-systolic dimension (mm)	40 (29–65)	43 (32–62)
Left ventricular ejection fraction (%)	46 (23–68)	39 (24–64)
Left ventricular end-diastolic volume (ml)	156 (112–234)	174 (147–242)
Left ventricular end-systolic volume (ml)	84 (54–188)	95 (62–188)
Mitral regurgitation grade (none/mild/moderate/severe)	14/11/3/3	15/16/10/6
Tricuspid regurgitation grade (none/mild/moderate/severe)	12/10/8/1	16/17/10/3
Tricuspid regurgitation pressure gradient (mm Hg)	30 (23–35)	24 (18–37)
Right ventricular diameter (mm)	38 ± 8	38 ± 9

Values are expressed as mean ± SD if the variable is normally distributed, median (interquartile range) if not, or number (percentage).

the test group and additional 49 for the validation group. Eleven patients were examined during mechanical ventilation. There was no significant difference in clinical characteristics between the 2 groups (Table 2).

In the test group, we found a close curvilinear relation between LS and RAP measured by pulmonary artery catheter (Figure 1). There was an excellent linear relation between ln [LS] and RAP measured by pulmonary artery catheter. Predicted RAP using the "best-fit" equation model correlated well with RAP (Figure 2). Bland-Altman plots revealed that the mean difference between predicted and actual RAP was −0.14 ± 1.77 mm Hg, with no tendency for a systematic bias (Figure 2). When the equation model derived from the test group was applied to the validation group, predicted RAP still correlated excellently with the actual RAP (Figure 2). Bland-Altman plots revealed that the mean difference between predicted and actual RAP averaged −0.05 ± 2.64 mm Hg, with no tendency for a systematic bias (Figure 2). When the equation model was tested in all 80 patients, correlation between predicted and actual RAP remained excellent (Figure 3). Bland-Altman plots revealed that the mean difference between predicted and observed RAP averaged −0.09 ± 2.33 mm Hg, with no tendency for a systematic bias (Figure 3). Notably, the relation persisted even in patients on mechanical ventilation or with severe tricuspid regurgitation ($r = 0.97$, $p < 0.0001$; Figure 3). Analysis of covariance did not show a significant difference between the equation model derived from both groups (RAP = −5.9 + 6.8 × ln [LS]) and the equation model developed in the test group (p = NS).

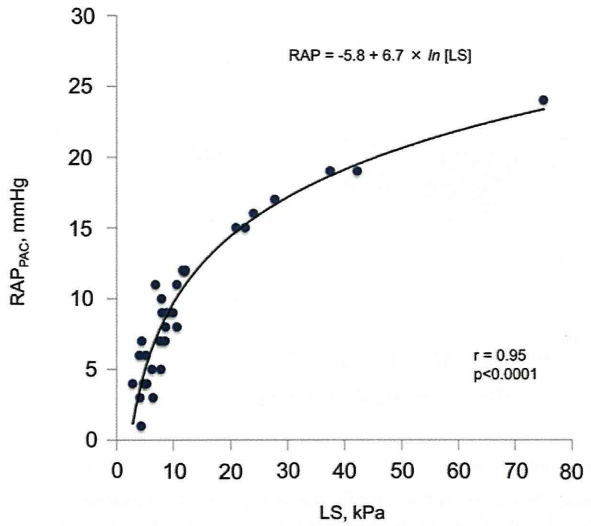

Figure 1. Relation between LS and RAP_{PAC} in the test group. Scatter plots showing the relation between the LS as assessed by transient elastography and RAP measured invasively with a pulmonary artery catheter in the test group. ln = natural log transformation; RAP_{PAC} = RAP determined using a pulmonary artery catheter.

test group. The ROC curve analysis of the IVC parameters showed a significant discriminatory ability to identify RAP >10 mm Hg (area under the curve = 0.800, 95% CI 0.604 to 0.913, p <0.0001). The corresponding optimal cut-off value was 15 mm Hg (range, 10 to 20). The area under the curve of LS for identification of RAP >10 mm Hg was 0.958 (95% CI 0.757 to 0.994, p <0.0001), which was significantly greater than that of the 2-dimensional echocardiographic IVC parameters (Figure 4). Based on the ROC curve analysis, the optimal cut-off value for LS for detection of RAP >10 mm Hg was an LS ≥10.6 kPa. The 2 methods for detecting elevated RAP were examined in the validation group (Table 3). The value of LS yielded a good balance among sensitivity, specificity, and accuracy in the validation group. Furthermore, LS ≥10.6 kPa showed a significantly greater sensitivity and accuracy for detecting RAP >10 mm Hg than echocardiography.

The intraclass correlation coefficients for intra- and inter-observer agreement were 0.997 (95% CI 0.993 to 0.999, p <0.0001) and 0.997 (95% CI 0.990 to 0.999, p <0.0001), respectively. Bland-Altman plot revealed no systematic bias (mean difference, 0.1 and 0.07, respectively).

The LS value weakly but significantly correlated with several hemodynamic, echocardiographic, and laboratory

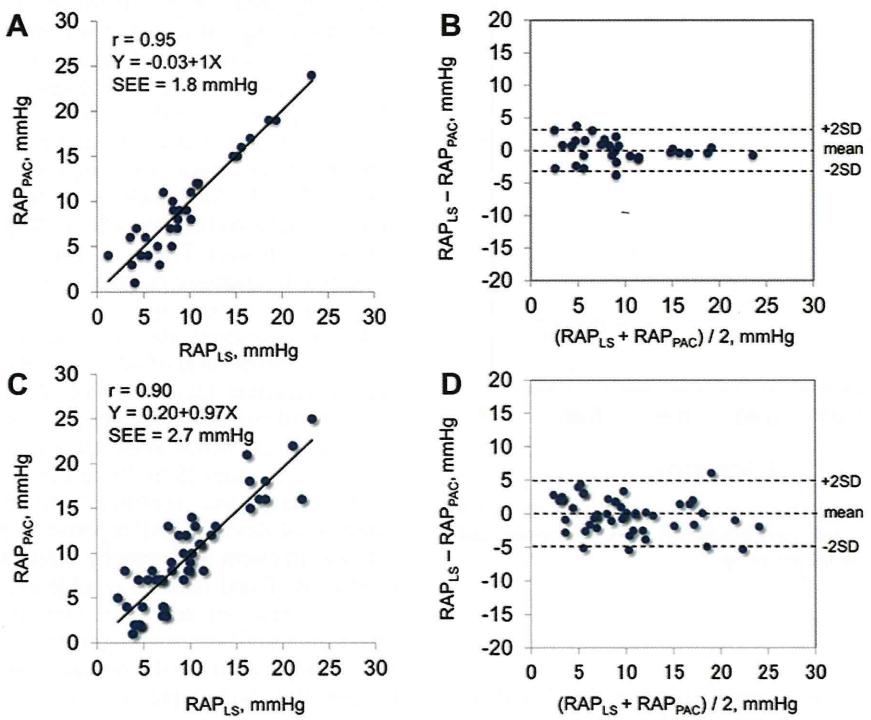

Figure 2. Relation between RAP_{LS} and RAP_{PAC} in the test and validation groups. Scatter plots showing the relation between RAP_{LS} and RAP_{PAC} in the test group (*A*). Bland-Altman plot: RAP_{LS} and RAP_{PAC} plotted against their mean values in the test group (*B*). Scatter plots showing the relation between RAP_{LS} and RAP_{PAC} in the validation group (*C*). Bland-Altman plot: RAP_{LS} and RAP_{PAC} plotted against their mean values in the validation group (*D*). RAP_{LS} = RAP estimated based on LS; RAP_{PAC} = RAP determined using pulmonary artery catheter; SEE = standard error of estimate.

Consistent with previous observations, RAP values estimated on the basis of IVC diameter and collapse (American Society of Echocardiography criteria) correlated significantly with actual RAP (r = 0.65, p <0.0001) in the parameters related to liver congestion (Table 4). However, stepwise multivariate analysis demonstrated that RAP was the only independent determinant of LS (standardized partial regression coefficient = 0.84, p <0.0001).

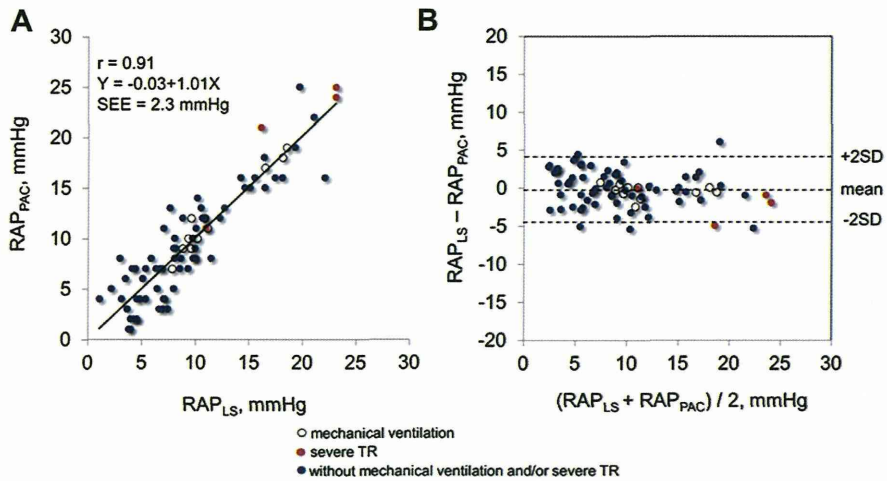

Figure 3. Relation between RAP_{LS} and RAP_{PAC} in all 80 patients. Scatter plots showing the relation between RAP_{LS} and RAP_{PAC} in all 80 patients (*A*). Bland-Altman plot: RAP_{LS} and RAP_{PAC} plotted against their mean values in all 80 patients with and without mechanical ventilation and severe tricuspid regurgitation (*B*). SEE = standard error of estimate; TR = tricuspid regurgitation.

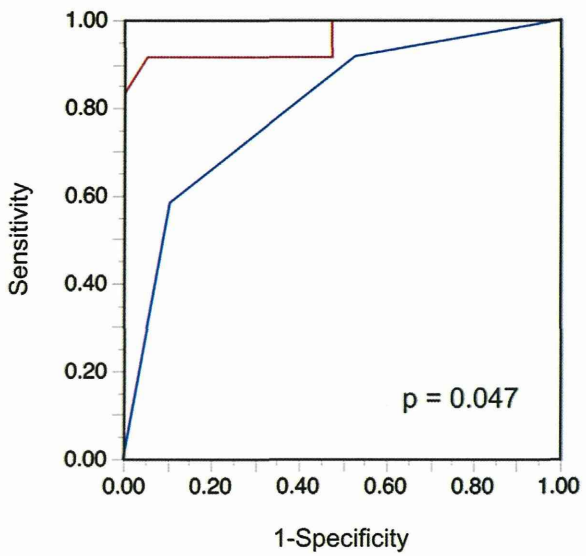

Figure 4. ROC curves for discrimination of RAP >10 mm Hg by LS measurement *(red line)* and echocardiography (IVC dimension and collapsibility) *(blue line)* in the test group.

Discussion

This is the first clinical study showing that LS measurement allows noninvasive and reliable estimation of RAP in patients with HF and no structural liver disease. There are 2 major findings in this study. First, we found a close correlation between RAP and LS values with a curvilinear regression equation. Second, LS showed a greater accuracy for detection of elevated RAP compared with conventional echocardiography and could also be applied to patients on mechanical ventilation and/or severe tricuspid regurgitation.

Several studies have investigated the influence of increased RAP on LS in HF.[13,14] One clinical study showed a modest correlation (r = 0.66) of LS with RAP estimated by echocardiography.[13] Another study reported increased LS in most patients with acute decompensated HF and absence of parenchymal liver disease. Furthermore, LS was shown to decrease considerably during medical treatment.[14] In parallel to our study, 21% of the cases had values above that normally used to diagnose cirrhosis (i.e., LS ≥12.5 kPa). Unfortunately, the study failed to precisely define the relation between LS and RAP because of a lack of hemodynamic data. The present study is the first to propose a regression model for estimation of RAP using the concept of transient elastography.

Echocardiography allows noninvasive estimation of RAP along with assessment of other important parameters such as cardiac function. However, IVC diameter and respiratory variation offer only semiquantitative assessment of RAP and may lead to erroneous inference, especially in patients with intermediate values (5 to 10 mm Hg).[8] Nevertheless, echocardiographic data confirmed the good specificity for detection of elevated RAP reported in previous studies.[11] In contrast, transient elastography offers a quantitative assessment of RAP and remains reliable even in patients on mechanical ventilation and with severe tricuspid regurgitation, where the use of echocardiography is usually limited.[8] Part of the study was to test the reproducibility of LS assessment. In agreement with a previous study on patients with liver cirrhosis,[15] we found excellent reproducibility of LS measurements in patients with HF.

Using hepatic vein flow patterns (i.e., the systolic filling fraction of the hepatic vein) was found to provide another noninvasive method to reliably and quantitatively assess RAP.[16] However, the method holds important shortcomings such as dependence of sonographer's skill and unavailability in patients with severe tricuspid regurgitation. In contrast,

Table 3
Accuracy, sensitivity, and specificity of liver stiffness (LS) and inferior vena cava (IVC) parameters for identification of right atrial pressure (RAP) >10 mm Hg in the validation group

Parameter	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Accuracy
Elastographic parameter					
LS ≥10.6 kPa (n = 49)	0.85*	0.93	0.89	0.90	0.90†
Echocardiographic parameter					
IVC diameter >21 mm + collapse <50% (n = 46)‡	0.56	0.86	0.71	0.76	0.74

* p = 0.046 versus IVC diameter >21 mm + collapse <50%.
† p = 0.049 versus IVC diameter >21 mm + collapse <50%.
‡ Estimated RAP according to the American Society of Echocardiography guideline algorithm.

LS measurement requires no learning curve, as previously described.[17] Our data show that the relation between LS and RAP remains reliable even in patients with severe tricuspid regurgitation.

There are several clinical conditions preventing reliable assessment of LS, that is, ascites, even if clinically undetected, narrow intercostal spaces, and morbid obesity (body mass index >40 kg/m^2).[15] It can also be problematic that many factors have been reported to affect LS such as all types of hepatitis, mechanical cholestasis, cellular infiltration, or deposition of amyloid.[5] In our patients, elevated LS mainly resulted from a high RAP, whereas the contribution of other laboratory parameters seemed relatively small. However, our findings indicate that these confounding factors should be taken into account when interpreting LS values in different clinical settings. There were only a small number of patients with severe HF and liver dysfunction due to HF in our cohort. Therefore, it is not conclusive whether our findings can be applied to critically ill patients. Caution should be exercised when applying our findings to continuous follow-up of patients as we designed a cross-sectional rather than a longitudinal study. For ethical reasons, we did not exclude parenchymal liver disease histologically by liver biopsy. However, we screened every individual by hepatic ultrasound. Although the results of the present study are promising, an interventional study comparing LS-guided treatment and standard methods (e.g., in intensive care unit patients) is encouraged to further elucidate the clinical implication of our findings.

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Table 4
Variables associated with liver stiffness (LS) in patients with heart failure

Variable	LS Value, Univariate	
	Correlation Coefficient	p Value
Age (yrs)		>0.1
Men		>0.1
Body mass index (kg/m^2)		>0.1
Body surface area, (m^2)		>0.1
New York Heart Association functional class, I/II/III/IV	0.46	<0.0001
Hemoglobin (g/dL)	−0.21	0.06
Platelet count (10^4/mm^3)	−0.48	<0.0001
Aspartate aminotransferase (IU/L)	0.47	<0.0001
Alanine aminotransferase (IU/L)	0.25	0.03
γ-Glutamyl transpeptidase (IU/L)	0.45	<0.0001
Alkaline phosphatase (IU/L)	0.31	0.006
Lactic dehydrogenase (IU/L)	0.46	<0.0001
Total bilirubin (mg/dl)	0.56	<0.0001
Total cholesterol (mg/dl)	−0.25	0.04
Triglycerides (mg/dl)	−0.41	0.0005
High-density lipoprotein cholesterol (mg/dl)	−0.26	0.03
Low-density lipoprotein cholesterol (mg/dl)		>0.1
Albumin (g/dl)	−0.36	0.001
Estimated glomerular filtration ratio (ml/min/1.73 m^2)	−0.29	0.01
B-type natriuretic peptide (pg/ml)	0.43	0.0002
Mean RAP (mm Hg)	0.90	<0.0001
Heart rate (beats/min)		>0.1
Systolic blood pressure (mm Hg)	−0.2	0.08
Diastolic blood pressure (mm Hg)		>0.1
Systolic pulmonary artery pressure (mm Hg)	0.36	0.001
Diastolic pulmonary artery pressure (mm Hg)	0.53	<0.0001
Pulmonary capillary wedge pressure (mm Hg)	0.63	<0.0001
Cardiac output (L/min)	−0.29	0.02
Cardiac index (L/min/m^2)	−0.35	0.002
Maximal IVC diameter (mm)	0.70	<0.0001
Minimal IVC diameter (mm)	0.75	<0.0001
Estimated RAP (mm Hg)	0.68	<0.0001
Left ventricular end-diastolic dimension (mm)		>0.1
Left ventricular end-systolic dimension (mm)		>0.1
Left ventricular ejection fraction (%)	−0.20	0.08
Left ventricular end-diastolic volume (ml)		>0.1
Left ventricular end-systolic volume (ml)	0.20	0.08
Mitral regurgitation grade (none/mild/moderate/severe)		>0.1
Tricuspid regurgitation grade (none/mild/moderate/severe)	0.38	0.0006
Tricuspid regurgitation pressure gradient (mm Hg)		>0.1
Right ventricular diameter (mm)	0.44	0.0002

Disclosures

The authors have no conflicts of interest to disclose.

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ORIGINAL ARTICLE

Liposomal Amiodarone Augments Anti-arrhythmic Effects and Reduces Hemodynamic Adverse Effects in an Ischemia/Reperfusion Rat Model

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Abstract
Purpose Although amiodarone is recognized as the most effective anti-arrhythmic drug available, it has negative hemodynamic effects. Nano-sized liposomes can accumulate in and selectively deliver drugs to ischemic/reperfused (I/R) myocardium, which may augment drug effects and reduce side effects. We investigated the effects of liposomal amiodarone on lethal arrhythmias and hemodynamic parameters in an ischemia/reperfusion rat model.
Methods and Results We prepared liposomal amiodarone (mean diameter: 113±8 nm) by a thin-film method. The left coronary artery of experimental rats was occluded for 5 min followed by reperfusion. Ex vivo fluorescent imaging revealed that intravenously administered fluorescent-labeled nano-sized beads accumulated in the I/R myocardium. Amiodarone was measurable in samples from the I/R myocardium when liposomal amiodarone, but not amiodarone, was administered. Although the intravenous administration of amiodarone (3 mg/kg) or liposomal amiodarone (3 mg/kg) reduced heart rate and systolic blood pressure compared with saline, the decrease in heart rate or systolic blood pressure caused by liposomal amiodarone was smaller compared with a corresponding dose of free amiodarone. The intravenous administration of liposomal amiodarone (3 mg/kg), but not free amiodarone (3 mg/kg), 5 min before ischemia showed a significantly reduced duration of lethal arrhythmias (18±9 s) and mortality (0 %) during the reperfusion period compared with saline (195±42 s, 71 %, respectively).
Conclusions Targeting the delivery of liposomal amiodarone to ischemic/reperfused myocardium reduces the mortality due to lethal arrhythmia and the negative hemodynamic changes caused by amiodarone. Nano-size liposomes may be a promising drug delivery system for targeting I/R myocardium with cardioprotective agents.

Keywords Liposome · Amiodarone · Lethal arrhythmia · Ischemia · Reperfusion

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Introduction

Therapies for the prevention and treatment of ischemia-induced life-threatening arrhythmias remain an unmet medical need [1]. Amiodarone is currently considered to be the most effective anti-arrhythmic drug available for treating life-threatening arrhythmias [2, 3], despite the fact that this compound has a negative impact on hemodynamic parameters [4, 5]. The intravenous administration of amiodarone is expected

to be beneficial for the immediate treatment of arrhythmias in emergency settings, such as acute myocardial infarction (AMI) [6, 7]. However, in clinical practice, the administration of amiodarone remains problematic for the treatment of AMI [8]. Although lower doses of amiodarone result in fewer incidences of death, high doses of amiodarone can cause hypotension and non-cardiac death, both of which may diminish the positive effects of amiodarone [8, 9]. Therefore, a novel delivery system is strongly desired to enhance the anti-arrhythmic effects of amiodarone without producing severe side effects.

Liposomes are widely used for drug delivery to actively or passively target specific organs and to improve drug stability in cancer and inflammatory diseases [10–12]. In ischemic/reperfused (I/R) myocardium, cellular permeability is enhanced and vascular endothelial integrity is disrupted [13, 14], suggesting that nanoparticles, such as liposomes, may be a promising drug delivery system for targeting I/R myocardium with cardioprotective agents [15]. Indeed, we have recently demonstrated that adenosine encapsulated by liposomes coated with polyethylene glycol (PEG) exhibited enhanced cardioprotective effects and attenuated side effects, such as hypotension and bradycardia, in an ischemia/reperfusion model of rats [16]. In the present study, we prepared liposomal amiodarone and examined 1) the targeted accumulation of liposomal amiodarone in the I/R myocardium, 2) the hemodynamic effects of the intravenous administration of liposomal amiodarone and free amiodarone, and 3) the anti-arrhythmic effects of these preparations in an I/R rat model. We showed that targeting the delivery of liposomal amiodarone to I/R myocardium reduces the mortality due to lethal arrhythmias and the negative hemodynamic changes caused by amiodarone in an I/R rat model.

Methods

Materials

The materials used to prepare PEGylated liposomes, including 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol) 2000 (DSPE-PEG2000), were kindly donated by Nippon Fine Chemical Co. (Takasago, Hyogo, Japan). Fluorescent beads (diameter 100 nm) were purchased from Invitrogen. All other materials were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male Wistar rats (9 weeks old and weighing 250–310 g; Japan Animals, Osaka, Japan) were used. The animal experiments were approved by the Osaka University Research Committee and were performed according to institutional guidelines. All studies conformed to the Guide for the care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Preparation of PEGylated Liposomes

PEGylated liposomes composed of POPC, DPPC, cholesterol, DSPE-PEG2000, and amiodarone were prepared by a thin-film method. Briefly, amiodarone and lipids dissolved in chloroform were evaporated to form a thin lipid film using a rotary evaporator. The lipid film was dried for at least 1 h under reduced pressure and then hydrated with PBS (pH7.4). The liposome solution was freeze-thawed for 3 cycles with liquid nitrogen. The particle size of the liposomes was adjusted by extrusion through 100-nm-pore polycarbonate filters (Nuclepore, Cambridge, MA, USA). The liposomal solutions were centrifuged at 453,000 g for 15 min (CS120GXL, Hitachi, Japan) to remove the untrapped amiodarone. Then, the liposomes were resuspended in PBS. To determine the efficacy of trapping amiodarone in the liposomes, an aliquot of the liposomal solution was solubilized with 1 % reduced Triton X-100 (Sigma-Aldrich), and the amount of amiodarone was optically determined at 240 nm.

Characterization of PEGylated Liposomes

The particle size and ζ potential of PEGylated liposomes diluted with PBS were measured by dynamic scatter analysis (Zetasizer Nano ZS; Malvern, Worcestershire, UK). The analyses were performed 15 times per sample, and the results represent the analysis of 3 independent experiments.

Experimental Protocol

Targeted Delivery of Fluorescent-labeled Nano-sized Beads to the I/R Myocardium

The rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Catheters were advanced into the femoral vein to infuse the drugs. Ischemia/reperfusion was induced by 5 min of left coronary artery occlusion followed by reperfusion [16]. After the hemodynamic parameters became stable, fluorescent-labeled nano-size beads, 100 nm in diameter (FluoSpheres, Invitrogen), were intravenously infused to the rats for 5 min before ischemia or before a sham operation (n=3, each). Fifteen minutes after reperfusion, the hearts were removed and cut into 5 sections parallel to the axis from the base to the apex. Then, ex vivo fluorescence images were obtained with an Olympus SZX12 stereoscopic microscope equipped with a DP71 digital camera (Olympus, Tokyo, Japan) before and after the hearts were sliced.

Targeted Delivery of Amiodarone and Liposomal Amiodarone to the I/R Myocardium

Catheters were advanced into the femoral artery and vein to measure the systemic blood pressure (BP) and to infuse the drugs into the anesthetized rats, respectively. Electrocardiographic and hemodynamic parameters, such as heart rate (HR) and BP, were continuously monitored during the study using a PowerLab system (ADInstruments, Castle Hill, Australia). After the hemodynamic parameters became stable, to clarify the targeted delivery of amiodarone and liposomal amiodarone to the I/R myocardium, we intravenously administered saline, free amiodarone (3 mg/kg) or liposomal amiodarone (3 mg/kg) to rats for 5 min before the onset of ischemia. Then, we obtained blood samples and myocardium from the I/R area.

Effects of Amiodarone and Liposomal Amiodarone on Lethal Arrhythmias

To evaluate the effects of amiodarone and liposomal amiodarone on lethal arrhythmias, we intravenously administered saline (n=7), free amiodarone (3.0 or 10.0 mg/kg) (n=6 each), PEGylated liposomes (empty liposomes) (n=6), and PEGylated liposomal amiodarone (3.0 mg/kg) (n=6) for 5 min before ischemia. The dose of amiodarone used in this study was lower than that used in a previous study [17] to clarify whether amiodarone encapsulated by liposomes coated with PEG exhibited enhanced anti-arrhythmic effects. Without any procedure such as electrical conversion or cardiac massage, ventricular tachyarrhythmias (VT/VF) occurred frequently during early period of reperfusion and the mortality of rats reached more than a half of cases in this model [18].

Measurement of Amiodarone Concentration

The concentration of amiodarone in serum and heart tissue from the I/R area was assayed by high-performance liquid chromatography (HPLC) as previously described [19]. The detection limit of the HPLC assay was 50 ng/mL. Blood and myocardial samples were obtained at the end of the experimental protocol. The sample preparation was performed as previously described [19]. Briefly, myocardium was freed from visible blood, thereafter rinsed with 0.9 % sodium chloride and stored at −20 °C until analysis. After that, myocardial tissue samples were finely minced and 100 mg were homogenized with 0.9 % sodium chloride (1 mL) and after centrifugation, the clear supernatant was injected into HPLC.

Quantitative Evaluation of Fluorescent-labeled Nano-sized Beads in the I/R Myocardium

To analyze the quantitative fluorescent intensity, signals from heart slices were quantified by image analysis (Image J; National Institutes of Health, USA) as previously described [20]. The signal intensity from the heart slices was evaluated as the average signals of the whole heart and the left ventricle (LV) (Fig. 2c).

Arrhythmia Analysis

The electrocardiographic tracings were independently analyzed by two of the authors, who were blinded to the treatment assignment. The duration of each spontaneous ventricular tachycardia or fibrillation episode during the I/R protocol was measured using the time scale provided by the recording software. Ventricular tachycardia was defined as 4 or more consecutive ventricular ectopic beats, and ventricular fibrillation was defined as a signal in which the individual QRS deflections could not easily be distinguished from one another. However, distinguishing ventricular tachycardia from fibrillation was often difficult [21]; therefore, we report ventricular tachycardia and fibrillation collectively as ventricular tachyarrhythmias (VT/VF) in this study. VT/VF duration and mortality were evaluated for 5 min of ischemia followed by 15 min of reperfusion.

Statistical Analysis

The parameters of the liposomes are expressed as the mean ± standard deviation (SD). Other data are expressed as the average ± standard error of the mean (SEM). To compare the parameters of the liposomes, unpaired t-tests were performed. We performed the Welch t-test to compare the amiodarone concentration in the plasma and myocardium. For hemodynamic parameters, the data were assessed with the paired t-test for comparisons to the baseline within a group. One-way repeated-measurement ANOVA followed by post-hoc Bonferroni's multiple comparisons were used for comparisons between groups. To address the differences in VT/VF duration among the groups, we performed a non-parametric (Kruskal-Wallis) test followed by evaluation with the Mann–Whitney U test. The mortality rates were compared using the Fisher's exact probability test. In all analyses, $P<0.05$ was considered to be statistically significant.

Results

Characterization of PEGylated Liposomes

We prepared 5 types of PEGylated liposomes composed of POPC, DPPC, cholesterol, and amiodarone. The ratio of unsaturated lipids (POPC) to saturated lipids (DPPC) varied (Fig. 1). During preparation of the liposomes, the POPC: DPPC:cholesterol:amiodarone molar ratio of 10:0:5:1 exhibited the best encapsulation efficiency for amiodarone compared with the other conditions (Fig. 1).