Tetrandrine reverses human cardiac myofibroblast activation and myocardial fibrosis

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Tetrandrine (TTD) is an emerging therapeutic for patients at risk of organ fibrosis. TTD has documented antihypertensive, antifibrotic, and anti-inflammatory effects (13). TTD is extracted from the root of *Stephania tetrandra* and has been used as an herbal therapy in Chinese medicine for hundreds of years. TTD has been administered to human patients for hypertension, cancer, liver fibrosis, and other diseases (11, 13, 15). To date, there are no clinical reports of TTD use for cardiovascular diseases in North America (16). In animal studies, TTD has shown beneficial effects on limiting cardiac myocardial remodeling in response to both pressure overload and coronary ischemia (4, 24, 25, 28, 29, 36, 37). In such models of human disease, cardiac fibrosis is attenuated in response to systemic TTD therapy (25). However, the utility and mechanisms underlying the effects of TTD on cardiac fibrosis are poorly understood. TTD is known to exert hemodynamic effects by lowering blood pressure though blockade of calcium channels (10, 13). It is unclear if TTD acts directly on the cardiac myofibroblast to influence its activity.

In the present study, we examined both in vitro and in vivo effects of TTD on TGF-β1-induced cardiac myofibroblast-mediated ECM remodeling and myocardial fibrosis. Using human cardiac myofibroblasts in three-dimensional (3D) collagen matrices, we examined the direct effects of TTD on cardiac myofibroblast activity and cell-mediated ECM remodeling. Using a rodent model, we assessed the effects of systemic TTD therapy on hypertension-induced cardiac fibrosis and the relationship of hemodynamics to antifibrotic effects.

**MATERIALS AND METHODS**

*Human cardiac myofibroblast isolation and expansion.* Right atrial appendages were obtained from consenting patients (N = 10) undergoing cardiac surgery at Foothills Medical Center (Calgary, AB). All experiments involving human tissue were approved by the Conjoint Health Research Ethics Board at the University of Calgary and conform to the Declaration of Helsinki. Biopsies were placed in Iscove’s modified Dulbecco’s medium (IMDM; Lonza, Walkersville, MD), washed in phosphate-buffered saline (PBS; Lonza, Walkersville, MD), minced into 1- to 3-mm fragments, and then plated in 100-mm Petri dishes (Falcon, Cornings, NY), with IMDM supplemented with 10% fetal bovine serum (Gibco by Life Technologies, Burlington, ON) plus 50,000 units of penicillin-streptomycin (Life Technologies, Burlington, ON). Explant tissue was cultured at 37°C in 5% CO2, with the medium replaced every 4 days. Cells were harvested by washing explants with PBS and then treating enzymatically with 3 ml trypsin for 3–5 min at 37°C.

*Characterization of human cardiac myofibroblasts by immunohistochemistry.* Cells were seeded on coverslips and fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and blocked in blocking buffer containing 2% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% bovine serum albumin. Cells were then permeabilized and stained for α-smooth muscle actin (Sigma-Aldrich, St. Louis, MO) and vimentin (Santa Cruz, Santa Cruz, CA) using the appropriate primary antibodies and secondary antibodies conjugated to Alexa Fluor 594 (Life Technologies, Burlington, ON). Cells were imaged using a Leica SP8 confocal microscope (Leica Microsystems, Richmond Heights, MO) and images were analyzed using Fiji (National Institutes of Health, Bethesda, MD) and ImageJ (National Institutes of Health, Bethesda, MD).

*Collagen synthesis.* Human cardiac explants were cultured in T-style flasks with culture medium (Iscove’s modified Dulbecco’s medium [IMDM]; Lonza, Walkersville, MD) plus 50,000 units of penicillin-streptomycin (Life Technologies, Burlington, ON) and treated with variable doses of TTD (by intraperitoneal injection (10, 13). It is unclear if TTD acts directly on the cardiac myofibroblast to influence its activity.

**CARDIAC FIBROSIS IS PRESENT** in most etiologies of end-stage heart disease and is associated with progressive cardiac dysfunction and clinical decompensation (6). Persistent myofibroblast activity and remodeling of myocardial extracellular matrix (ECM) can promote interstitial myocardial fibrosis, cardiac structural remodeling, myocardial dysfunction, and progression to heart failure (31). The triggers and coordinating factors that result in persistent myofibroblast activation in the failing heart are not fully defined, but transforming growth factor (TGF)-β1 is highly implicated (9). Myofibroblast-mediated myocardial fibrosis is an important therapeutic target for patients at risk of cardiac failure (2).

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Collagen gel contraction assay. Collagen gel contraction assay was performed as previously described (20). Five hundred microliters of IMDM, either alone (serum-free medium) or containing 10 ng/ml human recombinant TGF-β1 (Gibco by Life Technologies, Burlington, ON), with or without TTD (Sigma-Aldrich, St. Louis, MO) was added to each well. To determine whether TTD affected myofibroblast activity independent of Ca2+ influx or intracellular Ca2+ level, either a T-type calcium channel blocker nifedipin, L-type calcium channel blocker verapamil, or BAPTA-AM was added with TTD to the collagen matrix contraction model. Serial images of the collagen matrix dimensions were obtained from the time of release (baseline) and at 24, 48, and 72 h.

Cell isolation from collagen matrices. Cells were harvested from collagen matrices after 48-h culture and assessment of gel contraction. Cells were isolated by incubating the matrix in 100 μl of 500 U/ml collagenase type II (Worthington, Lakewood, NJ). Cells were then collected by centrifugation.

Flow cytometry. The cell pellet harvested was resuspended, and cells were fixed in ice-cold methanol followed by washing with PBS + 1% bovine serum albumin. Subsequently, cells were permeabilized by incubation with PBS + 1% Triton-X. An additional 2 ml of PBS + 1% Triton-X were added to the cell suspension, and cells were centrifuged at 2,000 rpm for 5 min. Cells were stained with anti-α-smooth muscle actin (SMA) (Abcam, Toronto, ON) conjugated with FITC (1:200 dilution; Sigma-Aldrich, St. Louis, MO). α-SMA expression was measured using a flow cytometer (LSRII, Becton Dickinson).

Annexin V-propidium iodide binding assay. Cells were resuspended in 500 μl of cold binding buffer. Cell suspensions were then stained with annexin V and propidium iodide (BD Pharmingen, Becton Dickinson, San Jose, CA), according to the manufacturer’s instructions. Annexin V expression and propidium iodide positive cells were quantified by flow cytometry.

Cell proliferation assay. Human cardiac myofibroblasts (20,000 in 80 μl of collagen matrix) were seeded into each well of a 96-well culture plate. In the presence of TGF-β1 (10 ng/ml; Gibco by Life Technologies, Burlington, ON) in serum-free medium, cells were treated with 0, 1, or 5 μM of TTD for 48 h. Cell proliferation was measured using WST-1 (Roche Life Science, Indianapolis, IN). Measurement of cell circularity was performed using the Multi-Cell Outliner plug-in for ImageJ (version 1.48, National Institutes of Health). Measurement of cell circularity was performed using the Multi-Cell Outliner plug-in for ImageJ. By this method, values approaching 0 are representative of round cell morphology, while values approaching 0.4 are considered increasingly nonround (5). Collagen fiber alignment index was calculated as the area under the curve bound by the peak intensity value ± 10°. Larger index values indicate increased ECM remodeling.

Rodent hypertensive model of left ventricular hypertrophy and fibrosis. The University of Calgary Health Science Animal Care Committee approved the experimental protocol. All procedures performed were in accordance with the Guide for the Care and Use of Experimental Animals from the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals (The National Academy Press, revised 1996). Male Dahl salt-sensitive (Dahl/SS) rats (N = 12) were ordered from Charles River Canada (Montreal, QC, Canada) at 5 wk of age. The animals were acclimatized for 1 wk and then, starting at 6 wk of age, exclusively fed a high-salt diet (AIN-76A with 8% NaCl; TestDiet, Richmond, IN) for the remainder of the study. At 12 wk of age, rats were randomly divided into four treatment groups of N = 3 each: TTD 0, PBS only; TTD 7.5 mg/kg TTD; TTD 15, 15 mg/kg TTD; and TTD 30, 30 mg/kg TTD.
Treatment for all groups was administered intraperitoneally twice a week for 4 wk. TTD was dissolved in 0.1 N HCl at a concentration of 15 mg/ml, and the resultant solution was neutralized with 0.1 N NaOH.

**Blood pressure assessments.** Blood pressure was measured by noninvasive tail cuff monitor (BP 2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC). After an acclimation period, blood pressure was measured and recorded once per week, and an average of 20 consecutive measurements were taken over 30 min.

**Myocardial compliance and quantification of fibrosis area.** At 16 wk of age, animals were euthanized, and hearts arrested in diastole and explanted. Passive pressure distention measures of the left ventricle (LV) were performed as previously described (18). In brief, a latex balloon connected to a closed fluid-filled system housing a pressure catheter was inserted into the LV, and volumes at 30 mmHg were recorded. LV was isolated and short-axis sections were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5-µm thickness, and stained with picrosirius red. Samples were imaged under light and polarized light microscopes. To quantify the fibrotic area, six non-overlapping random images from each heart section were captured using a digital camera (Olympus DP73) at ×200 magnification (Olympus BX 53 Microscopy). ImageJ software was used to quantify fibrosis as a percent collagen staining per image area.

**Statistical analysis.** All group data are presented as means ± SD. Data were obtained from a representative experiment of which each was repeated in triplicate. When only two groups were compared, Student’s t-test was performed. For comparison of more than two groups, one-way ANOVA was used and followed by appropriate post hoc comparison tests. All statistical analyses were performed using GraphPad Prism 6.0, with *P < 0.05* considered statistically significant.

**RESULTS**

**Characterization of human cardiac myofibroblasts.** Cell morphology was consistent with myofibroblasts. To further characterize the cells, we performed immunocytochemistry for fibroblast-specific markers (vimentin and fibronectin). Greater than 95% of the cultured cells stained positive for fibroblast markers (Fig. 1). Cells were also stained for other nonfibroblast cell types found in myocardium. Specifically, cells showed negative staining for von Willebrand factor (endothelial cells), troponin I (cardiomyocytes), and desmin (smooth muscle cells, skeletal muscle cells, cardiomyocytes) (Fig. 1).

**TTD attenuates human myofibroblast-mediated collagen gel contraction.** Measuring the extent of collagen gel contraction over time can elucidate the extent of myofibroblast activity and its influence on ECM remodeling (14). TGF-β1 was used to stimulate myofibroblast activity to induce collagen gel contraction (Fig. 2). TTD treatments at both 1 and 5 μM attenuated human cardiac myofibroblast-mediated collagen gel contraction compared with controls. TTD inhibited myofibroblast-mediated collagen gel contraction in a concentration-dependent manner over time (Fig. 2). We further explored if differences in gel contraction between groups were a result of altered cell

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Fig. 1. Characterization of primary human cardiac myofibroblasts. Greater than 95% cultured cells expressed vimentin (first panel) and fibronectin (second panel). Staining with an absence of von Willebrand factor (vWF; third panel), troponin I (fourth panel), and desmin (fifth panel) is shown, confirming these cells as myofibroblasts. Nuclei were stained blue with diamidinophenylindole. Objective ×40.
TTD reverses human cardiac myofibroblast activation

TTD inhibits human cardiac myofibroblast-mediated ECM remodeling. Using a novel floating nylon-grid mediated ECM remodeling system, myofibroblast activation and local ECM remodeling around isolated single cells was imaged using confocal microscopy and quantified by image analysis (Fig. 6). In the absence of TTD, human cardiac myofibroblasts stimulated by TGF-β1 were observed to have a stellate morphology with numerous cell extensions and local ECM remodeling around each extension (Fig. 6, A, B, and D). TTD altered the morphology of the cell toward a spherical shape in a dose-dependent relationship, and ECM remodeling around each cell extension was significantly attenuated (Fig. 6, A, C, and D), suggesting that TTD directly suppresses myofibroblast activation and myofibroblast-mediated ECM remodeling.

Calcium channel blockade does not mediate TTD effects on myofibroblast activity. Voltage-gated Ca²⁺ channels, known to exist in excitable cells, such as neurons and muscle cells, are also present in fibroblasts and may contribute to their activation (26, 30). TTD is a unique calcium channel blocker with specific inhibitory effects on L-type and T-type Ca²⁺ channels (13, 32). We explored if the effects of TTD on human cardiac myofibroblast-mediated collagen gel contraction was a consequence of calcium channel blockade. We assessed the effects of TTD in the presence and absence of a T-type calcium channel blocker, mibebradil (Fig. 7A) and an L-type calcium channel blocker, verapamil (Fig. 7B). Selective calcium channel blockade influenced but did not completely eliminate the effects of TTD on human cardiac myofibroblast-mediated collagen gel contraction. These data suggest that TTD effects in myofibroblast activation are not completely dependent on its calcium channel blockade activity. Given that other calcium channels are known to mediate myofibroblast activity, such as transient receptor potential channels (40), we further explored calcium channel signaling by using BAPTA-AM, a cell-permeant chelator, to control intracellular Ca²⁺. In the presence of BAPTA-AM, we continued to observe a suppressive effect of TTD on human cardiac myofibroblast-mediated collagen gel contraction (Fig. 7C). These data indicate that TTD influences human cardiac myofibroblast activity through mechanisms that are not dependent on calcium channel blockade or intracellular calcium.

TTD attenuates myocardial fibrosis and preserves LV compliance in hypertensive rats independent of hemodynamic load. We next evaluated the effects of systemic TTD administration in a clinically relevant rat model of hypertension-induced myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls. Myocardial hypertrophy, fibrosis, and failure (Fig. 8) were observed after treatment with escalating doses of TTD. Systolic blood pressure area under the curve was significantly reduced in animals treated with TTD compared with untreated controls, but was similar in all groups treated with TTD.
despite escalating doses (Fig. 8C). LV compliance was assessed by ex vivo passive pressure distention analysis and revealed a significant attenuation of myocardial stiffness with higher doses of TTD in the setting of equivalent hemodynamic load (Fig. 8C, TTD 7.5 mg/kg vs. TTD = 15 mg/kg). These data suggest that, at higher doses, TTD may act on myocardial ECM and tissue remodeling, independent of its influence on hemodynamic load.

**DISCUSSION**

Approximately one-half of all patients with heart failure have preserved systolic function and suffer primarily from diastolic dysfunction characterized by progressive myocardial fibrosis and chamber stiffness. Despite the prevalence of this condition, there is a fundamental lack of effective medical therapies or specific guidelines for effective treatments to
reduce the morbidity or mortality of heart failure with preserved ejection fraction (“diastolic heart failure”) (23). Hypertension is a common cause of progressive myocardial fibrosis with diastolic dysfunction, and the prognosis is similar to that of patients with systolic dysfunction (22). The discovery and validation of therapeutic targets that directly influence myofibroblast-mediated ECM remodeling are necessary to treat this common, yet poorly understood, cardiovascular problem.

For the first time, we document a direct influence of TTD on limiting human cardiac myofibroblast activation, ECM regulation, and ECM remodeling. We provide novel mechanistic data that these direct effects are not mediated through calcium channel blockade. This observation prompted us to explore the effects of TTD in vivo, as the known antihypertensive effects may not be primarily responsible for the antifibrotic effects of TTD on the heart. Using a clinically relevant hypertensive rat model of LV hypertrophy, we showed that TTD limits myocardial fibrosis and also prevents myocardial stiffening at higher doses, even between groups with similar hemodynamic loads. Collectively, these novel observations support a direct effect of TTD on cardiac myofibroblasts that significantly influences myocardial ECM and tissue remodeling.

TTD attenuates human cardiac myofibroblast activation and ECM remodeling. When plated on plastic under two-dimensional culture conditions, cardiac fibroblasts in vitro (within 48 h) undergo a phenotypic shift to myofibroblasts (5). Accordingly, our culture conditions result in a mixed population of cells that became >90% myofibroblasts with time, consistent with the observations of others (5, 17, 27, 33). Stimulated with exogenous TGF-β1, we assessed the differential effects of TTD

![Graphs and Data](http://ajpheart.physiology.org/Downloadedfrom)

**Fig. 4.** A: flow cytometry analysis for α-smooth muscle actin (SMA) expression in human cardiac myofibroblasts treated with TTD (N = 3). B: treatment with TTD significantly decreased the mean fluorescence intensity, **P < 0.001** by one-way ANOVA. C: messenger RNA levels were determined by quantitative RT-PCR and showed significant reductions after 1 and 5 μM TTD treatments (N = 3).
on activation of cultured human myofibroblasts within two different 3D collagen matrix platforms. TTD reversed the activation of human cardiac myofibroblasts in both of these models, and changes in ECM expression were observed to be a consequence of changes in myofibroblast activity toward a less active baseline state. Importantly, the observed decrease in myofibroblast activation occurred in the absence of exaggerated apoptosis or altered cell proliferation. We further explored the effects of TTD on human cardiac myofibroblasts using a floating (noncontractile) nylon-grid supported thin collagen gel culture platform system (19). This unique model allows myofibroblast activation and local ECM remodeling around isolated single cells to be assessed and compared between groups. We showed that increasing doses of TTD attenuated human cardiac myofibroblast activation, and, in so doing, preserved local ECM architecture and maintained ECM homeostasis.

**TTD mechanisms of action.** While TTD is a known calcium channel antagonist, the effects of TTD on myofibroblasts and ECM remodeling do not appear to be related to a hemodynamic effect alone. The evidence for this is TTD, when applied in vitro, suppresses myofibroblast activation and ECM remodeling. TTD interrupts the ability of TGF-β to induce collagen contraction when applied in vitro. TTD suppresses collagen synthesis and gene expression in vitro, and this is paralleled by less fibrosis in vivo. While TTD is a calcium channel blocker, we provide evidence that TTD suppresses myofibroblast activation in the presence of L-type and T-type calcium channel blockers. Calcium channel blockade with verapamil alone had a significant effect on myofibroblast activation, but this effect was increased in the presence of TTD. In addition, the use of the cytosolic calcium chelator BAPTA-AM suggests that a change in intracellular calcium does not completely explain TTD’s antifibrotic activity.

TTD is a unique calcium channel blocker with well-documented effects on limiting hypertension and fibrosis in animal models (4, 24, 25, 28, 29, 35–37, 39). These studies have not defined an antifibrotic mechanism for the observed effects on the heart, and the role of hemodynamic load as a mediator of the antifibrotic effects has not been compared or addressed. A direct mechanistic effect of TTD in fibroblasts is supported by studies that have examined the in vitro influence of TTD on human skin and subconjunctival fibroblasts, where suppressed SMAD signaling was observed (12, 41). TGF-β activates the SMAD-2,3 signal transduction pathway, and TTD may act to interrupt this signal transduction pathway.

**TTD prevents interstitial fibrosis and chamber stiffness in hypertensive rat model.** Likely as a consequence of reverting activated myofibroblasts toward an inactive baseline state, TTD has marked effects on regulating myocardial ECM remodeling. The observed in vitro effects of TTD on suppressing collagen expression and preserving ECM homeostasis were paralleled in vivo by decreased interstitial collagen area and reduced myocardial stiffness. We observed these effects in atrial-derived cells, and differences between atrial and ventricular-derived myofibroblasts have been documented. Atrial fibroblasts show a greater response to TGF-β in vitro and a more robust fibrotic response in vivo (3, 38). Further work should be performed to confirm our observations using human ventricular-derived cells. Our preliminary results suggest a similar response to TTD, as observed in atrial-derived cells (data not shown). Remodeling of myocardial ECM is dynamic.
and complex, involving changes in the amount of collagen, types of collagens, degree of collagen cross-linking, and numerous additional emerging pathways (6, 7). Importantly, changes in chamber stiffness can be influenced by both the quantity and quality of myocardial collagens (1). We observed a reduction in myocardial stiffness with higher doses of TTD, despite similar levels of collagen quantity and hemodynamic pressure loads between groups. These data may reflect preservation of ECM quality in addition to ECM quantity, resulting from a direct influence of TTD on reserving cardiac myofibroblast activity and, in so doing, preserving ECM homeostasis. These data support that TTD can influence myocardial fibrosis.

Fig. 6. A: confocal immunocytochemistry of human cardiac myofibroblasts (phalloidin stained; green) embedded in the 3D collagen matrix (autoreflectance; red) within a microgrid treated with varying concentrations of TTD. Morphological markers of the extent of myofibroblast activation were assessed as cell extension length (B) and cell circularity index (C) and showed attenuation of myofibroblast activation in response to increasing concentrations of TTD. D: collagen fiber alignment index demonstrates attenuation of ECM remodeling in response to increasing concentrations of TTD. Values are means ± SD. *P < 0.05.
and myocardial stiffness, independent of its effects on hemo-
dynamic load or calcium homeostasis, likely as a consequence
of direct effects on the myofibroblast. Progressive fibrosis and
chamber stiffening are the hallmarks of progression to heart
failure, and the effect of TTD documented in our study may
have clinical relevance.

**Clinical implications.** We believe that data from our in vitro
model has clinical relevance. Human 3D cell culture models
allow for improved translation to the human condition com-
pared with two-dimensional culture experiments using animal
cells. 3D matrix constructs best reflect in vivo tissue microen-
virons and permit normal physiological cell-ECM inter-
actions (8). Our unique floating gel system with rigid lateral
boundaries may best reflect in vivo physiological conditions as
it mimics the influence of basement membranes (19). In this
study, we used freshly isolated cardiac myofibroblasts from

![Graph A](image)

**Fig. 7.** Human cardiac myofibroblast-mediated 3D collagen gel contraction in response to TTD in the presence of T-type Ca\(^{2+}\)/H11001 channel blocker mibefradil (A), L-type Ca\(^{2+}\)/H11001 channel blocker verapamil (B), and a cell-permeant chelator BAPTA-AM (C). After blocking T- or L-type Ca\(^{2+}\) channels and binding intracellular Ca\(^{2+}\), 3D collagen matrix contraction was further decreased. Values are means \(\pm\) SD; \(N = 3\). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\), ****\(P < 0.0001\).

![Graph B](image)

**Fig. 8.** A: histologic specimens of left ventricular (LV) myocardium from untreated and TTD-treated hypertensive rats stained with picrosirius red imaged using polarized light (left) and light (right) microscopy, demonstrating decreased fibrosis in response to TTD treatment. B: interstitial collagen abundance within the LV myocardium of hypertensive rats is expressed as average %collagen per high-power field treated and was significantly reduced with TTD treatment at variable doses. *\(P < 0.05\). C: LV volumes as measured by passive pressure distension were matched between hearts at 30 mmHg and indexed to body weight. Systolic blood pressure area under the curve (SBP-AUC) measures are also shown (inset) as an index of hemodynamic load (TTD 0, \(N = 3\); TTD 7.5 mg/kg, \(N = 3\); TTD \(\geq\)15 mg/kg, \(N = 5\)). As assessed by SBP-AUC, hemodynamic load was decreased by TTD treatment but was not different between 7.5 and \(\geq\)15 mg/kg groups. However, passive LV volume was significantly different between the 7.5 and \(\geq\)15 mg/kg groups, despite similar hemodynamic load, indicating altered compliance with higher doses of TTD. Values are means \(\pm\) SD.
human hearts undergoing heart surgery, Santiago and coworkers (27) observed that cultured adult cardiac fibroblasts that differentiate to myofibroblasts in vitro share similar expression profiles to myofibroblasts found in vivo. We utilized an ECM composed of type I collagen, which is the primary isoform in the human heart (34). In addition, our in vivo rat model of human disease is clinically relevant, as myocardial ECM remodeling and fibrosis is induced by hypertension, a primary cause of human diastolic heart failure with progressive myocardial fibrosis and altered chamber compliance. 

Conclusions. TTD directly influences myofibroblast-mediated ECM remodeling with beneficial effects on myocardial fibrosis and preservation of chamber compliance. TTD should be explored as a potential therapy for patients with diastolic dysfunction secondary to pressure overload.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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