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Glycation and Serum Albumin Infiltration Contribute to the Structural Degeneration of Bioprosthetic Heart Valves

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Valvular heart diseases are associated with significant cardiovascular morbidity and mortality, and often require surgical and/or percutaneous repair or replacement. Valve replacement is limited to mechanical and biological prostheses, the latter of which circumvent the need for lifelong anticoagulation but are subject to structural valve degeneration (SVD) and failure. Although calcification is heavily studied, noncalcific SVD, which represent roughly 30% of BHV failures, is relatively underinvestigated. This original work establishes 2 novel and interacting mechanisms—glycation and serum albumin incorporation—that occur in clinical valves and are sufficient to induce hallmarks of structural degeneration as well as functional deterioration.

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**HIGHLIGHTS**

- Two novel and interacting mechanisms contributing to BHV SVD are reported: glycation and serum albumin infiltration.
- Glycation product formation and serum albumin deposition were observed in 45 clinical BHV explanted due to SVD as well as BHV subcutaneously implanted in rats.
- In vitro exposure to glycation and serum albumin elicited collagen network misalignment similar to that seen in clinical and rat explant BHV tissue.
- Glycation was sufficient to impair BHV hydrodynamic function in ISO-5840-compliant pulse duplication testing and concomitant serum albumin infiltration exacerbated these effects.

**SUMMARY**

Bioprosthetic heart valves (BHV) are widely used to treat heart valve disease. BHV leaflets are fabricated from glutaraldehyde cross-linked heterografts—predominantly bovine pericardium (BP) or porcine aortic valves. BHV are preferred over mechanical valves due to their technical versatility, adaptability to transcatheter valvular approaches, and freedom from lifelong anticoagulation therapy (1–3). However, BHVs are limited by degeneration of leaflet tissue matrix, termed “structural valve degeneration” (SVD). SVD, whose definition has recently been standardized (3), constitutes pathological modification of valve leaflets ultimately compromising biomechanical function. SVD occurs in all BHV recipients and is exacerbated in certain subpopulations, including patients with diabetes (4) and younger patients (5,6). BHV lifespans are limited by SVD (3,7) to an average of between 10 and 15 years. This process is driven by cellular, biochemical, and biomechanical mechanisms arising from valve properties, patient characteristics, and the interactions between them. Calcification is observed in the majority of SVD cases; however, approximately 30% of SVD cases are not associated with significant calcification (8,9). Further, observation suggests that calcification might be associated with SVD without necessarily being functionally causative (8). Several noncalcific mechanisms have been proposed to contribute to SVD, including inflammatory oxidation, tissue thickening, and collagen network degeneration (7,10); however, technologies to address known mechanisms thus far have not significantly improved valve durability, suggesting that additional key mechanisms remain unidentified (6).

Glycation is a complex constellation of nonenzymatic biochemical reactions involving the addition of sugar-derived moieties to protein nucleophilic groups. Glycation may involve biologically significant intermediates, such as Amadori products, and culminates in the formation of biologically irreversible advanced glycation end products (AGEs) (11–13). Glycation strongly contributes to structural and functional degeneration in various native tissues and diseases via 2 major avenues: 1) direct modification of extracellular matrix proteins via crosslinking and modification of protein interactions; and 2) modulation of cell phenotypes and instigation of inflammation via glycation product-mediated receptor signaling (14,15). Glycation elicits degeneration of collagenous native tissues via crosslinking and resultant disruption of collagen networks (16–18); yet, it has not been considered in the pathophysiology of BHV leaflets, for which collagen type I is the majority component (19,20). BHV lack living native cells or a functional surface barrier. Glycation in this context is
likely to occur primarily via infiltration from the surrounding blood of: 1) glycation precursors that modify the extracellular matrix structure directly; 2) pre-glycated proteins that deposit in BHVs; and 3) nonglycated proteins that are glycated in situ by infiltrated precursors. Therefore, we hypothesized that glycation and infiltration by human serum albumin (HSA), the most abundant and glycation-susceptible circulating protein (21,22), synergistically contribute to BHV SVD.

**METHODS**

**PATIENT POPULATION.** In order to establish clinical relevance for glycation and albumin infiltration in BHV, 45 patients with BHV aortic valve replacements requiring reoperation and BHV explantation were studied (Supplemental Table 1). Endocarditis was excluded. At initial operation, 25 patients (55.6%) underwent valve replacement only, 6 (13.3%) underwent valve replacement with aortic root replacement, 2 (4.4%) underwent valve replacement with ascending aorta repair, 2 (4.4%) underwent valve replacement concomitantly with coronary artery bypass grafting, and 10 (22.2%) underwent aortic valve replacement concomitantly with mitral valve repair. BHV durations ranged from 3.5 to 14.8 years (mean duration 8.6 ± 0.4 years). Echocardiography preceding reoperation demonstrated BHV aortic insufficiency in 33 patients (73.3%) and BHV aortic stenosis in 37 patients (82.2%). Comorbidities included aortic dilatation (26.7%), diabetes (17.8%), coronary artery disease (40.0%), hyperlipidemia (46.7%), hypertension (73.3%), smoking (33.3%), and bicuspid aortic valve (42.2%). Nearly one-half of patients (44.4%) had been treated with statins.

**IN VITRO GLYCATION/SERUM ALBUMIN EXPOSURE.** In order to model glycation and albumin infiltration in BHV tissue in vitro, 8-mm biopsy punches of BP were incubated in phosphate-buffered saline (PBS) (Corning, Corning, New York), PBS + 5% clinical-grade HSA (from stock 25% HSA, Octapharma via NOVA Biologics, Oceanside, California), PBS + 50 mmol/l glyoxal (from stock 88 mol/l glyoxal, Sigma-Aldrich, St. Louis, Missouri), or PBS + 50 mmol/l glyoxal + 5% serum albumin for 24 h, 2 weeks, or 4 weeks at 37°C.

**RAT SUBCUTANEOUS MODEL.** In order to model glycation and albumin infiltration in BHV tissue
in vivo, 8- to 10-mm discs of BP were subcutaneously implanted in 3-week-old Sprague-Dawley rats (n = 26; Charles River Laboratories, Wilmington, Massachusetts), with adherence to an approved protocol (AAAR6796). Each animal received 1 implant per subcutaneous pocket, with 4 to 6 implants per animal. Blood was drawn at the time of animal sacrifice via cardiac puncture and collected in EDTA blood tubes, after which it was immediately centrifuged at 4°C and 1,000 relative centrifugal force for 15 min. Plasma was aliquoted and stored at −80°C.

**Pulse Duplication.** In order to evaluate the functional effects of glycation and concomitant albumin infiltration in BHV, hydrodynamic pulsatile functionality of BHV was tested on a commercial, ISO 5840-compliant pulse duplicator (HDTi-6000, BDC...
Laboratories, Wheat Ridge, Colorado) with a PD-1100 pulsatile pump (BDC Laboratories). The flow, pulse rate, and driving waveform shape were controlled through Statys software (version 1.2) (BDC Laboratories). The pressure was adjusted via the Systemic Mean Pressure Control knob. Detailed experiment setup and calculation of all relevant parameters are described in the Supplemental Appendix.

RESULTS

CALCIFICATION, COLLAGEN MALALIGNMENT, GLYCATION, AND HSA INFILTRATION IN EXPLANTED BHV ARE ASSOCIATED WITH SVD.

With institutional review board approval (AAAR6796 [Columbia University] and 809349 [University of Pennsylvania]), surgical aortic valve replacement (SAVR) bioprosthetic explants were retrieved for this study (n = 45) from patients ranging in age from 34 to 86 years at the time of reoperation (mean 65.0 ± 13.6 years) (Supplemental Table 1, Supplemental Methods). A transcatheter aortic valve replacement (TAVR) bioprosthesis was also obtained and analyzed.

Calcification results (Supplemental Figure 1) showed an average leaflet calcification of 126 μg of calcium/mg of leaflet mass (SD = 107 μg/mg). Thirteen valves exhibited nearly no calcification (<10 μg/mg), 6 valves had intermediate calcification (between 10 and 100 μg/mg), and 26 valves had high calcification (>100 μg/mg). Unimplanted BHV were characterized with second-harmonic generation (SHG) microscopy, which demonstrated organized alignment of collagen fiber bundles (Figures 1A and 1B). Representative micro-computed tomography and SHG images of
explanted BHVs with various degrees of calcification are shown in Figures 1C and 1D. All explanted leaflets, regardless of calcification, showed disruption of collagen alignment by SHG (Figure 1E) compared with unimplanted BHVs (Figure 1B).

Clinical explants and unimplanted BHV biomaterials (glutaraldehyde-fixed BP and porcine aortic valve) were analyzed by immunohistochemistry (IHC) for generalized AGE, the AGE receptor ligand (CML) (23,24), the AGE cross-link glucoselane (25,26), and HSA (Figures 1F and 1G). Each of the 45 SAVR explants exhibited significant IHC staining for glycation products (Figure 1F) and HSA (Figure 1G) compared with unimplanted BHVs. IHC mean scores for all 45 explants are shown in Supplemental Figure 1A. Statistical analysis revealed no relationship of staining intensities to either calcification or the clinical determinants shown, such as diabetes mellitus (Supplemental Figure 1B). Collagen malalignment per SHG and positive AGE, CML, HSA, and glucoselane immunostaining were also noted for the TAVR explant (Supplemental Figure 1C). In BHV explants fabricated from BP, we observed uniform IHC staining for AGE throughout the tissue, whereas in BHV explants made from porcine aortic valve, IHC exhibited nonuniform staining with significant overlap among glycation products and HSA staining patterns (Figures 1F and 1G and Supplemental Figures 1D and 1E).

IN VITRO MODEL OF BHV INTERACTIONS WITH GLYOXAL AND HAS. To investigate the functional mechanisms of glycation and serum protein infiltration, an in vitro model using BP, 50 mmol/l glyoxal as a glycation precursor (12), and a physiological concentration (5% w/v) of clinical-grade serum albumin was designed. IHC on BP following 24 hours of incubation demonstrated glyoxal-generated CML staining and infiltration of HSA uniformly throughout the tissue (Figure 2A). Coincubation with glyoxal and HSA yielded increased CML staining compared with glyoxal alone (Figure 2A). 14C-glyoxal was used in order to measure the glycation capacity of BP. In a 28-day study, approximately 50% of the incorporated radioactivity seen at 28 days accumulated within the first 24 h (Figure 2B). In coincubation, 14C-glyoxal incorporation in BP in the presence of HSA was significantly less than without HSA (Figure 2B); however, this diminishment was expected as a result of inherent competition with the solid BP tissue by dissolved human-serum albumin for reaction with glyoxal. Tissue pre-incubated with HSA before incubation in glyoxal alone results in 14C-glyoxal incorporation comparable to the glyoxal-only condition without correction for any tissue mass increase caused by albumin incorporation. We then sought to visualize whether glycation and HSA infiltration affect the collagen microstructure of BP in vitro. In SHG images, BP samples exposed to either glyoxal or HSA for 28 days demonstrated collagen fiber bundle malalignment and relaxing of crimp compared with BP exposed to PBS. This effect was exacerbated in the presence of both glyoxal and HSA (Figures 2C and 2D) and comparable with SHG observations of clinical BHV (Figure 1E). To visualize HSA infiltration at the macromolecular level, transmission electron microscopy was performed. Electron micrographs (Figure 2E) showed longitudinal and cross-sectional views of collagen fibers from individual fiber bundles. BP incubated in 5% HSA with or without glyoxal exhibited an increase in interfibrillar particulates, whereas incubation with glyoxal alone did not. In addition, interfibrillar particulates appear closely associated with collagen fiber surfaces and demonstrate noncollagen fibrous aggregates in BP incubated with glyoxal.

RAT SUBCUTANEOUS EXPLANTS AND CIRCULATING BIOMARKERS REVEAL AGE-DEPENDENT CALCIFICATION AND GLYICATION OF BP. An established rat subcutaneous implantation model (Supplemental Figure 2A) of BHV calcification was used to investigate glycation in vivo and the impact of animal age in calcification and AGE-mediated SVD (27). Juvenile (3-week-old) and adult (8-month-old) rats received subcutaneous BP implants for either 7 or 30 days. Alizarin red staining (Figure 3A) revealed accumulation of calcium deposits within the explants in juvenile animals, which were more extensive in 30-day compared with 7-day explants. No calcification was detectable in adult animals, supporting the clinical observation of age-dependent calcification of BP in vivo (28,29). Quantification of calcium content in BP explants validated this observed increase in calcium accumulation in 30-day (160 ± 21.2 μg/mg) from 7-day explants (31.9 ± 6.8 μg/mg), both of which demonstrated greater calcium content compared with unimplanted BP (0.36 ± 0.06 μg/mg; p < 0.001) and to explants from adult animals (Figure 3B). Established circulating markers of calcification (PO4− [3.0 ± 0.19 μmol/ml vs. 2.4 ± 0.12 μmol/ml; p = 0.028]; alkaline phosphatase (ALP) [9.2 ± 1.5 μU/ml vs. 0.30 ± 0.13 μU/ml; p = 0.004]; and osteopontin (OPN) [33.9 ± 3.5 ng/ml vs. 9.3 ± 0.93 ng/ml; p < 0.001]) were also elevated in the plasma of juvenile rats when compared with adult animals (Figure 3C). SHG analysis of the rat explants (Figure 3D) revealed collagen network disruption in cross sections of both 7- and
30-day BP explants. The alignment coefficients of BP explants were significantly higher in juvenile than in adult animals (0.67 ± 0.02 vs. 0.43 ± 0.02; p < 0.001) (Figure 3E). In juvenile rats, the 30-day explants demonstrated a higher crimp distance compared with unimplanted BP (28.7 ± 0.40 mm vs. 25.2 ± 0.45 mm; p < 0.001), indicating the loss of characteristic collagen crimping. Overall, these results indicate progressive and age-dependent glycation and concomitant structural disruption of collagen alignment in BP tissue in the rat model. IHC (Figure 3F) revealed diffuse CML accumulation within both the 7- and 30-day BP explants that was more prominent in the juvenile animals. Plasma concentrations (Figure 3G) of soluble receptor for AGE (sRAGE) (3.7 ± 0.47 ng/ml vs. 1.7 ± 0.30 ng/ml; p = 0.007), methylglyoxal (3.9 ± 0.88 nmol/l vs. 1.3 ± 0.34 nmol/l; p = 0.020), and methylglyoxal protein adducts (6.8 ± 0.76 µg/ml vs. 1.3 ± 0.19 µg/ml; p < 0.001) were all increased in the juvenile rats compared with the adult cohort. IHC using an anti-HSA antibody (Figure 3H) that cross-reacts with rat albumin also indicated infiltration of albumin throughout the BP tissue by 7 days, with enhanced accumulation after 30 days. In contrast to all other plasma marker analyses, the plasma concentration of glycated albumin (Figure 3I) was lower in the juvenile rats (325 ± 45.3 pmol/ml) compared with the adult cohort (539 ± 35.4 pmol/ml).
p = 0.006). IHC also revealed diffuse staining for OPN (Supplemental Figure 2B) and AGE (Supplemental Figure 2C) in both 7- and 30-day implants, which was more pronounced in the juvenile animals. IHC for RAGE (Supplemental Figure 2D) demonstrated positive staining at the surface of 7- and 30-day explants from both juvenile and adult rats. IHC studies did not detect the presence of glucosepane in the subcutaneous explants (data not shown).

**IMPACT OF AGE ACCUMULATION ON BIOPROSTHETIC VALVE HYDRODYNAMIC PERFORMANCE.** To understand the susceptibility of intact BHV leaflets to glycation and HSA infiltration as well as to determine the roles of these mechanisms in degeneration of valve performances, we incubated 3 expired clinical-grade Carpentier-Edwards PERIMOUNT RSR BHVs (Edwards Lifesciences, Irvine, California) in PBS, 50 mmol/l glyoxal in PBS, and 50 mmol/l glyoxal plus 5% HSA in PBS, respectively, at 37°C for 35 days and evaluated their hydrodynamic function under physiological conditions. Hydrodynamic function of the valves was tested at 0, 1, 3, 7, 14, 21, 28, and 35 days of incubation using an ISO standard heart valve pulse duplicator system (test conditions in Supplemental Appendix). The baseline (time point “0”) values of mean pressure gradient and effective orifice area (EOA) of all 3 valves satisfied the requirements specified in ISO-5840, indicating the expected hydrodynamic performances of SAVR valves used in this study (Figures 4A to 4C). Both experimental in vitro incubation conditions resulted in a steady decline in EOA (Figures 4A to 4C) and increases in mean pressure gradient and peak jet velocity over time. Following 35 days of in vitro treatment, the BHV coincubated with glyoxal and HSA demonstrated a 17.5% decrease in EOA, 44.9% increase in mean pressure gradient, and 7.6% increase in peak jet velocity as compared with each of the baseline values. The BHV treated with glyoxal alone showed a 12.3% decrease in EOA, 27.1% increase in mean pressure gradient, and 5.0% increase in peak jet velocity as compared with its baseline values (Figures 4A to 4C). By comparison, the BHV incubated in PBS alone exhibited 4.6%, 15.9%, and 2.0% changes in these 3 parameters, respectively (Figures 4A to 4C and Supplemental Figure 3, Supplemental Table 2). Energy loss during each cycle was not significantly changed after 35-day PBS incubation (Figure 4D); however, glyoxal and HSA coincubation resulted in significantly worsened energy loss after 35 days (17.5 ± 0.23 J [baseline] vs. 30.6 ± 0.35 J [day 35]; p < 0.001) (Figure 4D). SHG imaging (Figure 4E) and analysis (Figures 4F and 4G) of valve leaflets after 35 days of treatments revealed collagen malalignment and the relaxation of collagen crimp following coincubated with glyoxal and HSA as compared with PBS or glyoxal. Similar to BP glycated in vitro, leaflet tissue of the valve treated with glyoxal alone was positively stained for CML, whereas the valve coincubated in glyoxal and HSA demonstrated leaflet accumulation of CML and HSA (Figures 4H and 4I). We investigated the significance of glycation and concomitant HSA infiltration to TAVR functionality using an in-house fabricated TAVR valve (Supplemental Figure 4A). Valve fabrication is described in the Supplemental Appendix. Similar to the observations in SAVR valves, TAVR valve also demonstrated collagen malalignment, decline in EOA, and increases in mean pressure gradient, peak jet velocity, and energy loss (Supplemental Figures 4B to 4G, Supplemental Table 2) as a result of coincubation with glyoxal and HSA. IHC assessments of HSA, AGE, and CML in our fabricated TAVR valve (Supplemental Figure 4C) showed similar results to those in clinical explants as well as our in vitro and in vivo studies.

**DISCUSSION**

Glycation is well-established as a functional mechanism of tissue degeneration in various diseases (13), yet this study is the first description of its involvement in the degeneration of BHVs. Similarly, whereas infiltration of circulating proteins on or in clinical (30–32) and in vivo (33,34) BHV tissue has been reported, this work establishes the relevance of serum albumin infiltration as well as interaction between protein infiltration and glycation in affecting BHV hydrodynamic performances. Our study employed a comprehensively translational approach, establishing clinical relevance via explant analyses (Figure 1), performing mechanistic modeling in vitro (Figure 2) and in vivo (Figure 3), and evaluating the functional significance of the mechanisms using a cardiac simulator (Figure 4).

IHC on 45 clinical explants indicate that accumulations of AGES and HSA are prevalent in failed BHVs and show collagen malalignment independently of the extent of calcification. The accumulation of AGES and HSA, together with the general lack of correlation with calcification, valve tissue type, or diabetes, suggest that glycation and albumin infiltration are fundamental mechanisms affecting all implanted BHVs. Although diabetes is logically expected to exacerbate glycation, we may have not been able to discern enhancement of glycation in a subpopulation of our failed valves, due to our IHC results indicating potentially saturated staining in general. Diabetes
may instigate earlier achievement of these high levels of glycation. Diabetic patient valves did tend to fail earlier (6.9 ± 2.1 years) than nondiabetic valves (9.0 ± 2.7 years) in our cohort, which skews toward early failures overall (8.6 ± 2.7 years). Our findings provide a mechanistic rationale explaining recent observations that SVD tends to occur earlier and more commonly in diabetic patients (4), who had glycation-related pathologies are highly exacerbated. Our results also inform understanding of the complex relationship between atherosclerosis-related changes and calcification in BHV. Atherosclerotic risk factors have been associated with SVD and atherosclerosis-like processes, such as lipid infiltration and inflammatory cell activation, are known to occur in BHV (3,7). Conflicting results have been reported regarding the effects of statin treatment on SVD and BHV calcification (35-37). Calcification has also been related to deposition of calcium-binding proteins in BHV. Although the protein deposition process generally involves endothelial barrier dysfunction and inflammatory cell-mediated events in atherosclerosis, the results reported here for albumin suggest that calcification-related protein deposition in BHV may be an atherosclerosis-unrelated, cell-free diffusive infiltration event. Nonetheless, glycation—particularly via glycated albumin—is also known to play roles in atherosclerosis-related processes via inflammatory cell activation (11,13,14). It is therefore reasonable to expect that glycation contributes to established inflammatory processes that occur on and in BHV. The experimental paradigms and hypotheses reported here provide a platform for further studies into the complex interactions of these mechanisms and co-factors in BHV SVD, which may explain the underpinnings of SVD in particular patient populations.

We sought to understand the mechanisms of AGE and HSA accumulation in BHVs by a glycation/protein infiltration in vitro model. The observed accumulation of AGEs and HSA throughout BP tissue after only 24 h of in vitro incubation implies that these processes begin impinging on BHV immediately upon implantation. Together, clinical correlation and in vitro assays suggest that HSA incorporation is enabled by glycation and/or that incorporated HSA increases the tissue’s capacity for glycation. The former possibility is supported by published data suggesting that glycation crosslinking permanently incorporates infiltrated albumin into solid tissue matrices (38-40). The latter possibility is informed by our clinical ex vivo and in vitro modeling observations, indicating preferential accumulation of glycation products at areas of HSA incorporation in clinical BHV and enhancement of tissue CML accumulation amid diminished overall glycation due to glyoxal exposure by coincubation with HSA. Glycation occurs primarily on lysine and arginine residues, while the majority of BHV tissue lysines are effectively sequestered from glycation by prior reaction with glutaraldehyde. Incorporation of infiltrated proteins, whose residues are generally unmodified, would increase the repository of glycation-susceptible lysines—as well as arginines—in the tissue. These observations suggest that human-derived serum albumin infiltration not only exacerbates accumulation of AGE in BHV, but also modifies the glycation profile toward lysine-directed AGE, which include the most prominent signaling AGE, CML (14,23,24), and the most abundant crosslinking AGE, glucosepane (25,26). Thus, the mechanistic crosstalk between glycation and HSA coincubation lead to the highest degree of structural alteration by SHG assessment and electron microscopy. Additionally, the incorporation of proteins may inculate BHV tissue with the properties of those proteins, such as calcium- and lipid-binding as well as high oncotic pressure in the case of HSA.

The rat model is an established method for testing biomaterial proprieties and calcification in vivo. This model resulted in calcium phosphate deposition within the central region of BP tissue (comparable to observations in clinical explants [41]), rather than on the surface, as noted with in vitro calcium-phosphate incubations (42). This system also allows modeling major risk factors for BHV failure, such as patient age. The assessments of both explants and circulating markers indicate a host-age dependence of tissue glycation, suggesting that enhanced glycation as well as calcification could contribute to accelerated SVD in pediatric patients. sRAGE derived from inflammatory cell turnover has been studied as a biomarker for cardiovascular disorders. RAGE is expressed on monocyte cell membranes, and RAGE ligand (such as CML) signaling can initiate monocyte-to-macrophage transition. Inflammatory cell aggregates in BHVs tend to be surface oriented, which is reflected by the hematoxylin counterstain of rat explants. It is possible that AGE formation in BHVs provides the opportunity for RAGE signaling and macrophage deposition to produce reactive oxygen species that result in OxA formation in BHV, as we demonstrated in both experimental and clinical BHV studies (8,43). The lack of demonstrable glucosepane in the rat explants may be due to short (30-day) implantation times.

Cardiac pulse simulators are required for hydrodynamic performance analysis of prosthetic heart valves under ISO 5840 standard and the U.S. Food and Drug Administration guidance. Thus, we aimed to provide functional evidence that AGE and HSA
accumulation weaken hydrodynamic performances of BHVs upon in vitro glycation. EOA, mean pressure gradient, and peak aortic jet velocity, were calculated to assess the hydrodynamic performances of BHV. All 3 parameters are important indicators for the clinical assessment of aortic valve stenosis severity (44,45). All in vitro incubation conditions resulted in a steady decline in EOA and increases in mean pressure gradients and peak jet velocities. These results suggest that the generation of AGEs in BHV significantly alters the biomechanical properties of valve leaflets, potentially causing leaflet stiffening. Additionally, BHV treated with glyoxal alone demonstrated less deterioration of hydrodynamic performances as compared with glyoxal and HSA coincubation. This could be attributed to the fact that HSA provides additional reactive sites for glyoxal and enhances AGE formation. Deterioration of hydrodynamic function in the forms of increased pressure gradients and jet velocities as well as decreased EOA is a definitive feature of the SVD pipeline and its diagnosis (3,46).

Together, the ubiquity of glycation product as well as serum albumin accumulation in failed clinical BHV and the demonstration of the sufficiency of glycation and concomitant albumin infiltration to degenerate BHV hemodynamic properties indicate that these synergistic mechanisms may be core elements driving noncalcific SVD in all BHV patients and in diabetic patients in particular (4).

**STUDY LIMITATIONS.** Our clinical series included only aortic valve replacements. Nevertheless, SVD mechanisms are comparable regardless of implant site (47,48). We restricted our study to a small group of representative glycation-related structures. AGE research has identified a myriad of moieties including numerous crosslinks and receptor ligands (11,12). The HSA used in our in vivo studies is a clinical grade, human isolated HSA to closely mimic in vivo albumin conditions. Assessing SVD in rat subcutaneous implants that lack exposure to systemic blood flow may be a critical consideration; however, our data show that HSA permeated throughout the implanted BP as in clinical BHV explants. There are also some limitations on our pulse duplicator assays including: 1) using physiological buffers rather than blood or fluids with viscosity approximating that of blood; and 2) the use of clinical BHVs with expired shelf-life dates, per Food and Drug Administration requirements, that could alter their susceptibility to glycation. However, the baseline hydrodynamic performances met the ISO-5840 standards. It should also be noted that the present studies did not include experiments involving inhibition of glycation, or the use of so-called “AGE-breakers” that have been shown to diminish AGE accumulation experimentally. These studies were not considered because none of the agents previously studied have been shown to be effective in clinical trials, and there are no approved antiglycation agents available for clinical use.

**CONCLUSIONS**

Overall, SVD is a multifactorial process that involves far more than the passive degeneration of leaflet materials. We propose that glycation and protein infiltration result in BHV tissue matrix disruption via multiple mechanisms: 1) reduction of BHV leaflet mechanical compliance due to AGE crosslinking; 2) enabling of the permanent incorporation of infiltrated proteins via AGE crosslinking (38-40); 3) modification of BHV leaflets by AGE and protein incorporation that can alter collagen fiber interactions and resultant force dissipation during biomechanical activity (16-18); and 4) proinflammatory responses to the valve leaflet tissue by signaling to receptors of glycation products, including the receptor for AGE (RAGE) (14,15,24). Thus, it is concluded that the accumulation of AGE and serum albumin in clinical explants and the impact of glycation on both the collagen fiber microstructure and on the hydrodynamic function of BHVs significantly contribute to SVD. Interactions of these mechanisms with other mechanisms and cofactors involved in SVD are being evaluated and will be reported in subsequent papers.

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**PERSPECTIVES**

**COMPETENCY IN MEDICAL KNOWLEDGE:** Advanced glycation end products together with serum protein infiltration are well-established contributors to diabetes and Alzheimer’s disease, and many other disorders. The association of this pathophysiology with bioprosthetic heart valve structural degeneration represents a novel disease mechanism that should be recognized and addressed.

**TRANSLATIONAL OUTLOOK:** Future clinical studies can identify high risk populations and study interventions to reduce the deleterious impact of advanced glycation end products and serum protein infiltration on bioprosthetic heart valve functionality.
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**KEY WORDS** advanced glycation end products, aortic valve disease, biomaterial, bioprosthetic heart valve

**APPENDIX** For an expanded Methods section and supplemental figures and tables, please see the online version of this paper.
Glycation and Infiltration of Serum Albumin
Implications for the Prevention of Bioprosthetic Valve Structural Degeneration*

Nancy Côté, BSc, PhD, Philippe Pibarot, DVM, PhD

The only available option for the treatment of severe aortic stenosis is aortic valve replacement (AVR) by a prosthetic valve using open heart surgery or transcatheter valve replacement. Over 200,000 AVRs are performed each year in the United States. The bioprosthetic valves (BPVs) that are most frequently (>90%) used to replace the native aortic valve are themselves subject to structural valve deterioration (SVD), which limits their durability and may lead to heart failure, valve reintervention, or death. About one-third of patients undergoing AVR with a BPV have evidence of SVD at 10 years. The long-term durability of BPVs is becoming an even more crucial issue nowadays, as there is currently a strong trend for implanting BPVs in younger patients with longer life expectancy. There is thus an important and urgent need to unravel the factors and mechanisms responsible for SVD.

Extracellular matrix deterioration, collagen fiber disruption, and fibrocalcific remodeling are the main pathobiological processes leading to SVD. For a long time, these processes have been considered as purely passive and degenerative. However, recent studies suggest that active, and potentially modifiable, processes, such as lipid infiltration, inflammation, immune rejection, and active mineralization may be involved in the pathogenesis of SVD.

In this issue of *JACC: Basic to Translational Science*, Frasca et al. (1) present the results of an elegant translational research study investigating the possible detrimental effects of infiltrated glycation products and human serum albumin (HSA) leading to disorganization of BPV tissue. The authors demonstrated the presence of infiltrated advanced glycation end products (AGEs) and HSA in: 1) 46 explanted BPVs; 2) BPV bovine pericardium incubated in vitro in glyoxal and HSA; and 3) rat subdermal BPV implants. Second harmonic generation microscopy revealed structural disruption of collagen fibers and organization of BPVs subjected to glyoxal and HSA incubation. Subsequent analysis of hemodynamic performance on a commercial heart valve pulse duplicator of unimplanted surgical and transcatheter BPVs exposed to glyoxal and HSA demonstrated reduction in valve effective orifice area and increased pressure gradient and peak jet velocity (17.48%, 44.86%, and 7.62%, respectively in 30-day exposure to glyoxal medium).

These results support the role of glycation and infiltration by HSA, the most abundant and glycation-susceptible circulating protein, in structural and functional degeneration of BPVs. These processes induce an adverse fibrotic remodeling of BPV tissues with disorganization of collagen fibers, leading to thickening and stiffening of valve leaflets (Figure 1).

Several studies reported a strong association between type 2 diabetes mellitus and BPV SVD (2,3), and one of the potential causal mechanisms that has been proposed to explain this association was the high
circulating levels of AGEs in patients with diabetes and their powerful pro-calcifying effect at the vascular and valvular levels. However, in the present study, the infiltration of AGEs and HSA within BPV leaflets resulted in fibrotic remodeling but with no significant calcification of BPV tissues. Moreover, in the small amount of patients with diabetes (17.8% of total explanted BPVs), no relationship was found between diabetes mellitus, BPV leaflet calcification, and the amount of infiltrated glycation products and HSA. These findings are counterintuitive because diabetes is an important factor contributing to production of AGEs and their receptor (receptor for advanced glycation end products [RAGE]), and hyperglycemia is a major determinant of albumin organ permeability in microcirculation and macrocirculation in patients with diabetes.

Younger age at the time of BPV implantation has been reported as one of the most important predictors of SVD and need for valve reintervention. Younger people generally have higher serum albumin concentration than do older individuals. Moreover, patients requiring AVR at a younger age most often have a bicuspid aortic valve. Some studies reported that circulating levels of AGEs are elevated in patients with bicuspid aortic valve, and particularly those with associated aortopathy (4). In the study by Frasca et al. (1), the proportion of patients with a native bicuspid valve at the time of BPV implantation was not reported, but this factor could have an effect on the infiltration of glycation products in implanted BPVs. In light of these findings, it is plausible that accumulation of glycation products and HSA within BPV tissues is higher in younger versus older patients, which could therefore contribute to explain the faster SVD observed in younger patients.

Although BPVs are produced of chemically treated tissues to prevent cell infiltration, prostheses implanted in the circulation often elicit cellular responses. Cells from the healing process of the host, migrating cells from the sewing interface of the valve, and cells from the bloodstream that attach to the cellular component of the tissue and the ultimately fixation will normally sequester all available tissue lysine. However, infiltrated proteins and cells found in BPVs could be a substrate for increased glycation and SVD development. In the future, a biological in vitro model that would study glyoxalation and HSA impact on explanted and degenerated BPVs could bring more evidence for an interaction and mechanisms between glycation products and infiltrated cellular component of the tissue and the ultimately applicable clinical responses to SVD.

During the past decade, transcatheter AVR has grown exponentially and is now rapidly expanding to low-risk populations. However, the long-term durability of transcatheter valves is still unknown, which is an important limitation in such population. The present study does not allow us to answer whether or not the durability as well as the mechanisms underlying SVD will be similar in transcatheter versus surgical BPVs, given that the majority of explanted BPVs in this study were surgical, with only 1 transcatheter valve. Further studies are needed to determine if there are any specifics in terms of factor or mechanism of SVD in transcatheter BPVs, which could translate into shorter or longer durability versus surgical BPVs.

One important clinical need is to develop blood biomarker of SVD that could be easily implemented in the clinical setting to identify patients at risk of SVD and to monitor the BPV structural and functional integrity during follow-up. In this context, the findings presented by Frasca et al. (1) raise the possibility that circulating levels of glycation products could serve as an early and sensitive marker of SVD, as well as of the risk of BPV failure and reintervention. This potential clinical implication of the results of Frasca et al. (1) needs to be validated in longitudinal cohorts of patients with aortic BPV.

There is, unfortunately, no medical treatment that can prevent or stop the progression of SVD, and our knowledge on the mechanisms leading to SVD is still largely incomplete. The study of Frasca et al. (1) cast some light on a novel mechanism that could be targeted by pharmacological therapies or by lifestyle modification. Indeed, the severity of AGE-RAGE-mediated cardiovascular disease can generally be attenuated with decreased AGEs consumption, suppression of RAGE, and increased levels of soluble receptor of RAGE (sRAGE). The reduction in the AGEs levels can be achieved by reduction in consumption of food containing AGEs, low temperature cooking, and shorter duration of cooking (Figure 1). AGE production can be reduced with drugs such as statins, metformin or telmisartan or by vitamins. Statins, angiotensin converting enzyme inhibitors, rosiglitazone, vitamin D, and physical activity can increase sRAGE levels.
(antagonist of RAGE-AGE effects), whereas curcumin is able to reduce RAGE (5). Exogenous administration of sRAGE can also have a beneficial impact on cardiovascular disease. In light of the results of Frasca et al. (1), these pharmacotherapeutic strategies should also be considered and tested in patients with BPV in order to prevent or slow SVD. One advantage of the context of BPV versus native aortic valve is that the treatment can be instituted immediately after BPV implantation, before any SVD process has yet started. Hence, randomized controlled trials using anti-glycation therapies can be envisioned following AVR, with the goal of primary prevention of SVD.

Another future perspective for avoiding the deleterious effect of AGEs and HSA in infiltration on BPV tissues would be to modify the design of the BPV per se, including, for example: 1) polymer coating at the surface of the BPV leaflet to prevent infiltration of glycation products and HSA; and 2) chemical and physical processes to stabilize the extracellular macromolecular components of the BPV tissues and therefore avoid deterioration related to infiltrated glycation products.

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**KEY WORDS** aortic stenosis, bioprosthetic valves, end glycation valves, human serum albumin, structural valve deterioration
Circulating Progenitor Cells in Patients With Coronary Artery Disease and Renal Insufficiency

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 Patients with established coronary artery disease (CAD) and renal insufficiency (RI) (estimated glomerular filtration rate <60 ml/min/1.73 m²) are at an increased risk of adverse cardiovascular (CV) outcomes (1,2). Novel factors that contribute to this increased risk in patients with CAD and renal insufficiency are of considerable research interest. Circulating progenitor cells (CPCs) are mononuclear cells derived primarily from the bone marrow that contribute to vascular repair and regeneration largely through paracrine mechanisms (3-5). Circulating progenitors can be measured as mononuclear cells expressing the cluster of differentiation (CD) 34 epitope and these cells have the potential to differentiate into endothelial, hematopoietic, and nonhematopoietic (mesenchymal, lacking CD45 expression) phenotypes (6,7). CD133 is a 5-transmembrane antigen seen on primitive stem cells that is lost during cellular maturation, and coexpression of CD34 with CD133 (CD34+/CD133+) identifies a hematopoietic CPC-enriched subpopulation (8,9). Coexpression of chemokine (C-X-C motif) receptor 4 (CXCR4) with CD34 (CD34+/CXCR4+) characterizes cells with capacity for tissue repair via homing of CPCs to SDF-1 (stromal-derived factor 1)-enriched hypoxic environments (10). Although CD34+/CD133+ and CD34+/CXCR4+ subtypes are enriched for hematopoietic progenitors, coexpression of vascular endothelial growth factor receptor 2 (VEGF2R) with CD34 (CD34+/VEGF2R+) identifies a rarer subpopulation of CPCs enriched for endothelial progenitors (11).

Previous work from our group has shown that presence of “traditional” CV risk factors including diabetes, hypertension, hypercholesterolemia, and smoking early in life is associated with higher CPC...
counts \(12,13\). This stimulation is likely a result of mobilization of progenitors from the bone marrow in response to the risk factor-mediated injury, and represents activation of the endogenous regenerative or reparative systems \(5,13,14\). It is worth noting that continuous exposure to risk factors with age leads to depletion in CPC counts \(14\). In this context, the association of renal insufficiency, a “nontraditional” CV risk factor, with CPC counts and the impact of age on this association has not been evaluated to date.

Furthermore, prior research from our group and others has shown that lower levels of CPCs are independently associated with a higher risk of adverse CV outcomes \(15\-18\), and small studies have reported similar findings in patients with end-stage renal disease \(19\-22\). Nonetheless, the predictive value of CPCs in patients with CAD and renal insufficiency, a high-risk group, has not been previously studied. Therefore, we sought to investigate:

1) the association between renal insufficiency, age, and CPC counts; and 2) the predictive value of CPCs among patients with CAD and renal insufficiency. We hypothesized that renal insufficiency would be inversely associated with CPC counts, and that lower CPC counts would contribute to the increased risk of adverse events in patients with renal insufficiency.

**METHODS**

**STUDY POPULATION.** Participants enrolled in the Emory Cardiovascular Biobank, a prospective registry of patients undergoing cardiac catheterization for evaluation of CAD at 3 Emory Healthcare-affiliated hospitals were enrolled in this study \(23\). All participants provided written informed consent, and the study was approved by the Emory University Institutional Review Board. Subjects with acute myocardial infarction, severe valvular heart disease, organ

### Table 1: Baseline Characteristics of Participants Stratified by Renal Insufficiency Status

| Characteristic                                      | Overall (N = 1,281) | No Renal Insufficiency (n = 835) | Renal Insufficiency (n = 446) | p Value |
|----------------------------------------------------|---------------------|---------------------------------|-------------------------------|---------|
| eGFR, ml/min/1.73 m²                                | 68.0 ± 26.7         | 83.4 ± 15.7                     | 39.1 ± 17.6                   | <0.001  |
| Age, yrs                                           | 65.5 ± 13.1         | 63.4 ± 12.3                     | 69.5 ± 13.4                   | <0.001  |
| Male                                               | 776 (60.6)          | 517 (61.9)                      | 259 (58.1)                    | 0.187   |
| Black                                              | 283 (22.1)          | 162 (19.4)                      | 121 (27.1)                    | 0.002   |
| Diabetes                                           | 513 (40.1)          | 296 (35.5)                      | 217 (48.7)                    | <0.001  |
| Current smoking                                    | 60 (4.7)            | 47 (5.6)                        | 13 (2.9)                      | 0.036   |
| Hypertension                                       | 1,156 (90.5)        | 731 (87.9)                      | 425 (95.3)                    | <0.001  |
| Hypercholesterolemia                               | 951 (74.2)          | 618 (74.0)                      | 333 (74.7)                    | 0.841   |
| Body mass index, kg/m²                             | 29.4 ± 6.4          | 29.2 ± 6.4                      | 29.6 ± 6.5                    | 0.216   |
| Hemoglobin, g/dl                                   | 13.0 ± 1.9          | 13.5 ± 1.8                      | 12.3 ± 1.8                    | <0.001  |
| White blood cells, cells/µl                        | 6,734 ± 1,793       | 6,640 ± 1,749                   | 6,909 ± 1,861                 | 0.015   |
| CAD history                                        | 1,054 (82.3)        | 685 (82.0)                      | 369 (82.7)                    | 0.818   |
| HF history                                          | 451 (35.2)          | 252 (30.2)                      | 199 (44.6)                    | <0.001  |
| PAD history                                        | 246 (19.2)          | 129 (15.4)                      | 117 (26.2)                    | <0.001  |
| Ejection fraction, %                               | 53.0 (12.8)         | 54.0 (12.1)                     | 51.1 (13.8)                   | <0.001  |
| High-sensitivity troponin I, pg/ml                  | 6.5 (3.4-15.3)      | 5.0 (2.9-10.2)                  | 12.1 (6.1-27.1)               | <0.001  |
| B-type natriuretic peptide, pg/ml                  | 103.9 (43.9-270.0)  | 74.9 (33.9-175.1)               | 210.7 (91.2-662.7)            | <0.001  |
| ACE inhibitor/ARB use                               | 662 (51.7)          | 420 (50.3)                      | 242 (54.3)                    | 0.178   |
| Aspirin use                                        | 1,006 (78.5)        | 667 (79.9)                      | 339 (76.0)                    | 0.116   |
| Beta-blocker use                                   | 937 (73.1)          | 588 (70.4)                      | 349 (78.3)                    | 0.003   |
| Clopidogrel use                                    | 488 (38.1)          | 299 (35.8)                      | 189 (42.4)                    | 0.022   |
| Statin use                                         | 895 (69.9)          | 571 (68.4)                      | 324 (72.6)                    | 0.125   |
| CD34+ , cells/ml                                    | 1,637 (1,034-2,494) | 1,688 (1,080-2,501)            | 1,507 (698-2,485)             | 0.025   |
| CD34+/CD33+ , cells/ml                             | 747 (451-1,019)     | 756 (468-1,121)                 | 741 (418-1,184)               | 0.116   |
| CD34+/CXCR4+ , cells/ml                            | 794 (485-1,341)     | 801 (519-1,382)                 | 762 (430-1,231)               | 0.012   |
| CD34+/VEGFR2+ , cells/ml                           | 41 (12-138)         | 47 (13-146)                     | 36 (12-112)                   | 0.052   |
| Cardiovascular death/MI                             | 175 (14.0)          | 86 (10.5)                       | 89 (20.4)                     | <0.001  |
| All-cause death                                    | 234 (18.7)          | 116 (14.2)                      | 118 (27.1)                    | <0.001  |

Values are mean ± SD, n (%), or median (interquartile range). Ejection fraction, high-sensitivity troponin I, and B-type natriuretic peptide were measured in 1,198,1,251, and 1,130 participants, respectively.

ACE = angiotensin-converting enzyme; ARB = angiotensin II receptor blocker; CAD = coronary artery disease; CD = cluster of differentiation; CXCR4 = C-X-C chemokine (C-X-C motif) receptor type 4; eGFR = estimated glomerular filtration rate; HF = heart failure; MI = myocardial infarction; PAD = peripheral artery disease; VEGF2R = vascular endothelial growth factor receptor 2.
transplantation, immunosuppressive medication use, leukocytosis (defined as white blood cell count >11,000 cells/μl), and active infection, inflammatory disorder, or cancer were excluded. A total of 1,281 subjects (446 with renal insufficiency) were analyzed in this study, and the data supporting our findings are available from the corresponding author upon reasonable request.

**PARTICIPANT CHARACTERISTICS.** Participants were interviewed to collect information about demographic characteristics, smoking history, medical history, and CV medication (angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker, aspirin, beta-blocker, clopidogrel, and statin) use as previously described (23). The prevalence of diabetes, hypertension, hypercholesterolemia, and established CV disease subtypes (CAD, heart failure [HF], and peripheral artery disease) was determined by physician diagnosis or treatment (23). Medical records were reviewed to extract ejection fraction information and confirm self-reported medical history. Weight and height were measured at enrollment, and body mass index was calculated by dividing weight by height (kg/m²). Complete blood count, including hemoglobin level measured as g/dl and white blood cell count measured as cells/μl, was measured at enrollment (23). Serum creatinine was measured at enrollment as well (23), and estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation (24). Participants with eGFR <60 ml/min/1.73 m² (GFR categories G3a, G3b, G4, and G5) were grouped together and classified as patients with renal insufficiency (25). Data regarding albuminuria were not collected. Circulating levels of high-sensitivity troponin I (hsTnI) and B-type natriuretic peptide (BNP) were measured using the Abbott ARCHITECT analyzer (Abbott Laboratories, North Chicago, Illinois) (26).

**CPC ASSAYS.** CPCs were measured in peripheral arterial blood samples collected in EDTA tubes before contrast administration for cardiac catheterization (17). Blood samples were prepared within 4 h of collection and incubated with fluorochrome-labeled monoclonal antihuman mouse antibodies to identify surface markers expressed on mononuclear cells before quantification using flow cytometry. A total of 300 μl of peripheral blood was incubated with 7 μl of FITC-CD34 (BD Biosciences, San Jose, California), PerCP-CD45 (BD Biosciences), PE-VEGFR2 (R and D Systems, Minneapolis, Minnesota), 5-μl APC-CD133 (Miltenyi, Bergish Gladbach, Germany), and 3-μl PE-Cy7-conjugated anti-CXCR4 (clone 1265; EBioscience, San Diego, California) in the dark for 15 min (27). Then, 1.5-ml ammonium chloride lysing buffer was added to lyse red blood cells, following which 1.5-ml staining medium (phosphate-buffered saline with 3% heat-inactivated serum and 0.1% sodium azide) was added to stop the lysing reaction (27). Prior to flow cytometry, 100 μl of AccuCheck Counting Beads (Cat#: PCB100; Invitrogen, Carlsbad, California) were added to act as an internal standard for direct estimation of the concentration of target cell subsets (27). At least 2.5 million events were acquired from the cytometer. Flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, Oregon) with filter set at CD45med+ cells. This selection excludes CD45bright (lymphoblasts) and CD45− (non-hematopoietic progenitor) cells. CPC counts (CD34+, CD34+/CD133+, CD34+/CXCR4+, and CD34+/VEGF2R+) were measured using CD45med+ filter and are reported as cell counts per milliliter (Supplemental Figure 1) (27). A subset of 20 samples
was analyzed on 2 occasions by 2 technicians, and percent repeatability coefficients (%) were calculated as the standard deviation of differences between pairs of measurements/mean of measurements × 100. The repeatability coefficients were 2.9%, 4.8%, 6.5%, and 21.6% for CD34⁺, CD34⁺/CD133⁺, CD34⁺/CXCR4⁺, CD34⁺/CD133⁺/CXCR4⁺, and CD34⁺/VEGF2R⁺, respectively (12).

**FOLLOW-UP AND OUTCOMES.** Study participants were prospectively followed for 2 outcomes of interest, a composite of CV death and nonfatal myocardial infarction (MI) events, and all-cause mortality. Follow-up data were available for 1,253 subjects (436 with renal insufficiency) and were obtained using annual phone contact, electronic medical record review, and the Social Security Death Index and state records (23). The cause of death was determined from medical record review or by direct contact with the participants’ family member(s). Cardiovascular death and nonfatal MI events were adjudicated by 2 independent cardiologists blinded to study data (23). Cardiovascular death was defined as death...
attributable to an ischemic CV cause such as fatal MI, stroke, or sudden death secondary to a presumed CV cause in this high-risk population (23). Nonfatal MI events were adjudicated using the third universal definition of MI (28).

**STATISTICAL ANALYSIS.** Participant characteristics were reported as frequency and percentage for categorical variables and as mean ± SD or median (interquartile range) for continuous variables depending on distribution. Differences between subjects with and without renal insufficiency were evaluated using the chi-square test for categorical variables and using the independent-sample Student’s t-test or Mann-Whitney U test, as appropriate, for continuous variables. Baseline characteristics were also described across CPC count categories.

CPC counts were non-normally distributed and were analyzed as continuous variables after log-transformation (log_{cell count}/0.0001). The association of renal insufficiency, eGFR, and participant characteristics with CPC counts was assessed using unadjusted linear regression models. The impact of aging on the association of renal insufficiency with CPC counts was evaluated in age- and multivariable-adjusted linear regression models by exploring the multiplicative interaction between renal insufficiency and age dichotomized at the median value (70 years) for participants with renal insufficiency. Beta coefficients in linear regression models were exponentiated to transform them to the linear scale. A similar approach was used to study the association of dialysis treatment with CPC counts.

The association of renal insufficiency with adverse outcomes in the overall cohort was studied using Cox proportional hazards regression models adjusted for age, sex, race, diabetes, current smoking, hypertension, hypercholesterolemia, body mass index, hemoglobin, white blood cell count, CAD history, heart failure history, peripheral artery disease history, angiotensin converting enzyme inhibitor or angiotensin-II receptor blocker use, aspirin use, beta-blocker use, clopidogrel use, and statin use as covariates. Stepwise Cox regression using backward elimination with model removal p threshold of 0.10 was used to analyze all covariates.

**TABLE 3** Association Of Circulating Progenitor Cell Counts With Adverse Outcomes Among Patients With Renal Insufficiency

|              | CD34⁺ | CD34⁺/CD13³⁻ | CD34⁺/CXCR4⁺ | CD34⁺/VEGF2R⁺ |
|--------------|-------|--------------|--------------|---------------|
|              | HR (95% CI) | p Value | HR (95% CI) | p Value | HR (95% CI) | p Value | HR (95% CI) | p Value |
| CV death/MI  |       |              |              |              |              |         |
| Univariable  | 1.44 (1.20-1.73) | <0.001 | 1.27 (1.16-1.38) | <0.001 | 1.38 (1.16-1.65) | <0.001 | 1.00 (0.94-1.06) | 0.972 |
| Multivariable| 1.30 (1.08-1.56) | 0.006 | 1.28 (1.16-1.43) | <0.001 | 1.28 (1.06-1.54) | 0.009 | 0.98 (0.91-1.04) | 0.478 |
| All-cause mortality |       |              |              |              |              |         |
| Univariable  | 1.35 (1.14-1.58) | <0.001 | 1.22 (1.12-1.33) | <0.001 | 1.26 (1.08-1.48) | 0.004 | 0.98 (0.93-1.04) | 0.507 |
| Multivariable| 1.18 (0.99-1.39) | 0.058 | 1.20 (1.08-1.33) | 0.001 | 1.09 (0.92-1.10) | 0.320 | 0.97 (0.92-1.03) | 0.335 |

Multivariable Cox models included log-transformed CPC count, eGFR, age, sex, race, diabetes, current smoking, hypertension, hypercholesterolemia, body mass index, hemoglobin, white blood cell count, CAD history, HF history, PAD history, ACE inhibitor/ARB use, aspirin use, beta-blocker use, clopidogrel use, and statin use as covariates. Stepwise Cox regression using backward elimination with removal p threshold of 0.10 was utilized to analyze covariates in all Cox models, following which the independent variable of interest was added to the model. Association of lower CPC counts (log_{2} cell count transformed, reflecting a 50% relative decrease in count) with CV death or MI and all-cause mortality in the overall cohort and the subgroup of patients with renal insufficiency was studied using multivariable-adjusted Cox models, and results were reported as hazard ratio (HR) and 95% confidence interval (CI). The improvement in risk discrimination afforded by CPC counts was studied using the integrated discrimination index (IDI). In a sensitivity analysis, Fine and Gray competing-risks regression models were used to analyze the CV death or MI outcome and all-cause mortality was treated as a competing event. In a second sensitivity analysis, multivariable-adjusted Cox models were further adjusted for circulating levels of hsTnI and BNP.

CPC subtypes associated with outcomes were dichotomized at their respective median counts and participants were categorized into those without renal insufficiency, and those with renal insufficiency and CPC counts above or below the median. The association of these categories with outcomes was analyzed using multivariable-adjusted Cox models and the survival function of participants in these categories was plotted. All analyses were performed using SPSS Statistics Version 25 (IBM Corporation, Armonk, New York), SAS Version 9.4 (SAS Institute, Cary, North Carolina), and R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). A 2-tailed p value < 0.05 was considered statistically significant.
RESULTS

Baseline characteristics of study participants are described in Table 1. The mean age was 66 ± 13 years, 39% were women, 21% were black, and almost 35% (446) of the cohort had renal insufficiency, with 216 in category G3a (eGFR 45 to <60 ml/min/1.73 m²), 114 in G3b (eGFR 30 to <45 ml/min/1.73 m²), 37 in G4 (eGFR 15 to <30 ml/min/1.73 m²), and 79 in G5 (receiving dialysis and eGFR <15 ml/min/1.73 m²). Subjects with renal insufficiency were older (median age 70 years), more frequently black, and had a higher prevalence of diabetes, hypertension, heart failure, peripheral artery disease, beta-blocker use, and clopidogrel use, and higher white blood cell count and lower hemoglobin levels as compared with those without renal insufficiency (Table 1). Baseline characteristics of participants stratified my median CPC counts are described in Supplemental Tables 1A-D. Participants with higher counts for all CPC types were relatively younger and more frequently men.

ASSOCIATION OF RENAL INSUFFICIENCY WITH CPC COUNTS. The predictors of CPC counts in the overall cohort are listed in Supplemental Table 2. As reported previously, age and peripheral artery disease had strong inverse associations, while male sex and higher hemoglobin levels were positively correlated with all CPC counts (12,29). eGFR, as a continuous variable, correlated positively with CD34+, CD34+/CD133+, and CD34+/CXCR4+ counts, but not with CD34+/VEGF2R+ counts in unadjusted analyses (Supplemental Table 2). Thus, renal insufficiency was associated with a 10% to 11% lower CD34+, CD34+/CD133+, and CD34+/CXCR4+ counts in unadjusted linear regression models (Table 2), and no multiplicative interaction with CV medication use was seen (all p for interaction > 0.10).

The relationship of renal insufficiency with CPC count was attenuated after adjustment for age (Table 2, model 1). Differences in CPC counts among participants with and without renal insufficiency across age percentiles are reported in Supplemental Figure 2. A consistent multiplicative interaction between renal insufficiency and median age (70 years) for CD34+ (p = 0.007), CD34+/CD133+ (p = 0.006), CD34+/CXCR4+ (p = 0.044), and CD34+/VEGF2R+ (p = 0.055) counts was observed. Thus, among older participants (>70 years of age), presence of renal insufficiency was associated with a 14% to 35% lower CD34+, CD34+/CD133+, CD34+/CXCR4+ and CD34+/VEGF2R+ CPC counts, even after adjustment for age and other demographic and clinical characteristics, while no association was observed among those <70 years of age (Figure 1, Table 2). Similar results were observed for CD34+ and CD34+/CXCR4+ counts when participants were divided based on dialysis treatment (Supplemental Table 3).

OUTCOMES WITH RENAL INSUFFICIENCY. In the overall cohort, 175 CV death or MI and 234 all-cause death events were recorded during a median follow-up of 3.5 (interquartile range 1.5 to 5.2) years. In multivariable-adjusted Cox models, participants with renal insufficiency compared with those without were at a 48% higher risk of CV death or MI and 39% increased risk of all-cause mortality (Supplemental Table 4). The association of renal insufficiency with
adverse outcomes was not attenuated after adding CPC counts to Cox models, and lower hematopoietic CPC counts (CD34+, CD34+/CD133+, and CD34+/CXCR4+) were associated with higher CV death or MI and all-cause mortality in the overall cohort (Supplemental Table 4).

**CPC COUNTS AND OUTCOMES IN RENAL INSUFFICIENCY.** Among patients with CAD and renal insufficiency, a total of 89 CV death or MI events and 118 all-cause deaths were observed. CD34+, CD34+/CD133+, and CD34+/CXCR4+ counts were lower among participants that experienced a CV event as compared with those who did not (Supplemental Figure 3). Thus, lower CD34+, CD34+/CD133+, and CD34+/CXCR4+ counts were associated with higher CV death or MI and all-cause mortality rates in unadjusted Cox models (Table 3). In multivariable-adjusted models, the association between hematopoietic CPC counts and CV death or MI remained significant, while only CD34+/CD133+...
counts were independently associated with all-cause death (Table 3). No associations between CD34+/VEGF2R+ counts and outcomes were observed (Table 3). There were no interactions of hematopoietic CPC counts with age or with dialysis use (all p for interaction > 0.05) for both outcomes.

Age, eGFR, current smoking, heart failure history, and hemoglobin level were independent predictors of CV death or MI (Supplemental Table 5). Addition of CD34+, CD34+/CD133+, or CD34+/CXCR4+ counts to a baseline model comprising of these predictors results in significant improvements in IDI (0.181; 95% CI: 0.007 to 0.320; p = 0.040; 0.201; 95% CI: 0.042 to 0.309; p = 0.028; and 0.151; 95% CI: 0.004 to 0.254; p = 0.040; respectively) for CV death or MI. The independent predictors of all-cause death included age, eGFR, race, heart failure history, hemoglobin, and angiotensin converting enzyme inhibitor or angiotensin-II receptor blocker use (Supplemental Table 5). However, addition of CPC counts did not improve IDI for all-cause death.

Patients with coronary artery disease, renal insufficiency, and (A) CD34+, (B) CD34+/CD133+, or (C) CD34+/CXCR4+ counts below the respective median cutoffs were at a higher risk of all-cause mortality. Patients with coronary artery disease and renal insufficiency but circulating progenitor cell counts above the respective median cutoffs were at a similar risk of all-cause mortality as those without renal insufficiency. Abbreviations as in Figure 1.
First, in sensitivity analyses, the association of CPC counts with CV death or MI events was similar to Cox models when the Fine and Gray competing-risks regression approach was used (Supplemental Table 6). Second, hsTnI (HR: 1.10; 95% CI: 0.94 to 1.28; p = 0.223) and BNP (HR: 1.07; 95% CI: 0.89 to 1.28; p = 0.481) were not associated with CV death or MI in Cox regression models, while an independent association with all-cause death was seen with both hsTnI and BNP (HR: 1.15; 95% CI: 1.01 to 1.31; p = 0.039; and HR: 1.19; 95% CI: 1.02 to 1.39; p = 0.028, respectively). Importantly, further adjustment for hsTnI or BNP did not change the association of CPCs with outcomes (Supplemental Table 7).

The risk of CV death or MI and all-cause mortality was similar among participants without renal insufficiency and those with renal insufficiency and high (≥median values) hematopoietic CPC counts (Table 4, Figures 2 and 3). In comparison, only participants with renal insufficiency and lower (<median values) hematopoietic CPC counts were at a 75% to 80% higher risk of CV death or MI and 57% to 63% higher risk of all-cause death as compared with those without renal insufficiency (Table 4). Similar associations were observed when Fine and Gray competing-risks regression models were used to analyze the CV death or MI outcome (Supplemental Table 8).

**DISCUSSION**

In this large cohort study of patients with CAD, we found that the presence of renal insufficiency was associated with lower CPC counts among older participants (>70 years of age) after adjusting for differences in demographic characteristics, risk factors, and medication use. We also demonstrated that low CPC count, indicative of impaired endogenous regenerative capacity, was an independent predictor of adverse CV outcomes among participants with renal insufficiency. In contrast, patients with renal insufficiency and higher CPC counts, indicative of preserved regenerative capacity, had similar outcomes as those without renal insufficiency. These observations suggest that the relatively poor outcomes in patients with CAD and renal insufficiency are related, at least in part, to impairment of endogenous regenerative capacity.

**RENAL INSUFFICIENCY AND CPC COUNTS.** Previous studies investigating the relationship between renal disease and CPC counts in patients without known CAD have reported 4% to 30% lower levels of CPCs in those with end-stage renal disease compared with healthy control subjects (30–36). Potential mechanisms underlying the lower CPC counts in renal disease include: 1) exposure to uremic toxins including beta(2)-microglobulin, indoxyl sulfate, and indole-3 acetic acid that inhibit CPC chemotactic mobility and promote their apoptosis (37,38); 2) induction of a permanent defect in bone marrow hematopoietic stem cell niche (39); 3) impaired PC mobilization and proliferation because of renal insufficiency-associated systemic inflammation and oxidative stress, demonstrated as an inverse association between CD34+/VEGF2R+ cells and circulating tumor necrosis factor-alpha and interleukin-6 levels among patients receiving hemodialysis (40); and 4) inhibitory effects of sera of patients with end-stage renal disease on CPC differentiation and migration (31) and effects of lower erythropoietin levels contributing to CPC deficiency in these patients (41).

The association of renal insufficiency with CPC counts in a large cohort of high-risk patients with CAD has not been well characterized. In a smaller study of 102 patients with stable angina and CAD, those with renal insufficiency were reported to have lower VEGF2R-expressing CPCs, but this study did not include women and the analyses were not adjusted for relevant confounders (42). In our study, we were adequately powered to investigate the independent association between renal insufficiency and CPC counts in patients with CAD. We found that this relationship is age-dependent, and the presence of renal function impairment is independently associated with lower CPC counts, but only in the older age group. This novel observation is concordant with previous studies that have shown that presence of CV risk factors at a relatively young age is associated with either normal or higher CPC counts, reflecting an endogenous reparative response to subclinical vascular injury related to risk factor exposure (12,13). This mobilization of CPCs into the peripheral circulation is exhausted at an older age after continued exposure to injurious stimuli (14). Overall, our results indicate that relatively young patients with CAD and renal insufficiency have similar CPC counts as those with CAD alone, whereas differences in CPC counts between the 2 groups become apparent at an older age.

**CPC COUNTS AND OUTCOMES IN PATIENTS WITH CAD AND RENAL INSUFFICIENCY.** Previous studies have shown that low CPC counts are associated with CV events in patients with CAD (15–18) and in patients receiving dialysis treatment for end-stage renal disease (19–22). For instance, in a seminal 2015 meta-analysis, Rigato et al. (18) demonstrated that low CPC counts are associated with ~2-fold risk of CV events among patients with CAD. Maruyama et al. (19)
showed that a low CD34+ count is associated with a 2.2-fold risk of CV events in patients receiving chronic hemodialysis. Our findings are consistent with these prior observations, as we observed that low hematopoietic CPC counts among patients with CAD and renal insufficiency are associated with ~1.8-fold risk of CV events. Herein, we have demonstrated that CPC counts impact prognosis in patients with CAD and renal insufficiency, even before end-stage renal disease develops.

Our study participants underwent extensive phenotyping for 4 different CPC subtypes, and our results indicate that low counts of cell subtypes enriched for hematopoietic progenitors, CD34+; CD34+/CD133+, and CD34+/CXCR4+ cells, impact prognosis in this population. Previous research has shown that human CD133+ cells are protective in murine models of acute tubular and glomerular damage (43), and administration of CD133+ cells stimulates erythropoietic production and limits renal fibrosis after acute kidney injury (44). Additionally, SDF-1, the primary chemokine responsible for CXCR4+ cell mobilization from the bone marrow, is expressed in the normal kidney, and its expression is enhanced 24 h after renal ischemia (45,46). Taken together, these studies provide some pathophysiologic insight regarding the association of higher CD34+/CD133+ and CD34+/CXCR4+ counts with favorable prognosis in patients with CAD and renal insufficiency.

Last, we have also demonstrated that patients with renal insufficiency and high CPC counts have similar outcomes as those without renal insufficiency, indicating that an important mechanism underlying the increased CV risk in patients with CAD and renal insufficiency is impaired endogenous regenerative capacity that can be measured using CPC counts. Important implications of our results are that reduced regenerative capacity is a novel risk factor in patients with CAD and renal insufficiency and may be used as a biomarker to identify those at highest risk. Whether cell-based therapies would improve CV outcomes in this population remains to be investigated (45,47,48). Paradoxically, most clinical trials tend to exclude this high-risk population from CV cell therapy trials.

STUDY STRENGTHS. Strengths of our study include the large number of participants studied and the detailed exploration of the relationship between renal insufficiency and various CPC subtypes. Study participants were followed for more than 3 years for adjudicated CV events, and the independent association of CPC subtypes with outcomes was determined.

STUDY LIMITATIONS. Potential limitations of the study are the use of estimated eGFR using the Chronic Kidney Disease Epidemiology Collaboration equation, which, like all other creatinine-based estimating equations, remains imprecise. We did not investigate the association of CAD or renal insufficiency duration with CPC counts in this study. The functional activity of CPCs was not evaluated. However, previous studies have shown impaired PC function in renal disease (30,33). Finally, the observational nature of this analysis does not imply causation.

CONCLUSIONS

In conclusion, we found that renal insufficiency is independently associated with lower CPC counts among older patients with CAD. Lower CPC counts in patients with CAD and renal insufficiency are independently associated with an increased risk of adverse CV events, whereas those with preserved CPC counts have similar risk as those without renal insufficiency. Therapeutic interventions targeting endogenous regenerative capacity in this high-risk patient population are warranted.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Our study demonstrated that renal insufficiency is associated with impaired regenerative capacity, measured as decreased counts of CPCs, in older patients with coronary artery disease. Low CPC counts are independently associated with an increased risk of CV events and can stratify future CV risk in patients with CAD and renal insufficiency.

TRANSLATIONAL OUTLOOK: Future research studies should focus on deciphering the mechanistic basis of the association of renal insufficiency with impaired regenerative capacity in older patients with CAD. Randomized controlled trials to study the prognostic impact of therapeutic interventions that augment CPC counts in patients with CAD and renal insufficiency are warranted.
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**KEY WORDS** coronary artery disease, outcomes, progenitor cells, regenerative capacity, renal insufficiency

**APPENDIX** For supplemental tables and figures, please see the online version of this paper.
Circulating Progenitor Cells Predict Clinical Outcomes in Patients With Coronary Artery Disease and Renal Insufficiency*

Nikolaos I. Vlachogiannis, MD,a,b Konstantinos Stellos, MD,a,b

Bone marrow-derived CD34\(^+\) cells have been used extensively to reconstitute the hematopoietic system after radiation or chemotherapy; however, their regenerative potential has attracted research interest in their broader therapeutic capacity for tissue healing post-injury. Increasing experimental and human evidence shows that CD34\(^+\) circulating progenitor cells (CPCs) are integral for cardiovascular resilience through recruitment to sites of injury and in vivo differentiation into endothelial cells. Recent experimental evidence supports that CD34\(^+\) cells may induce angiogenesis in animal models of myocardial or peripheral ischemia. Injection of human CD34\(^+\) cells in the ischemic limb of diabetic mice has been shown to accelerate healing of ischemic tissue and vessel growth, whereas systematic administration of human CD34\(^+\) cells led to more efficient tissue repair in a mouse model of renal ischemia/reperfusion injury through synthesis of proangiogenic cytokines. Interestingly, we have previously shown that human CD34\(^+\) cell adhesion onto vascular wall is integral for the reduction of neointima formation in a mouse model of vascular injury (1). Although how CD34\(^+\) CPCs exert so many beneficial effects remains a matter of debate, the consensus is that their number in blood is associated with tissue-healing processes.

In this issue of *JACC: Basic to Translational Science*, Mehta et al. (2) address the prognostic value of CPCs in patients with coronary artery disease (CAD) and renal insufficiency. In a large cohort, consisting of 1,253 patients undergoing cardiac catheterization for evaluation of CAD (including 436 patients with renal insufficiency) followed up for a median of 3.5 years, the authors elegantly quantitated CPCs using flow cytometry and examined their prognostic value for cardiovascular (CV) death or nonfatal myocardial infarction (MI) as well as all-cause mortality. CPCs were defined as CD45\(^{\text{med}}\) cells expressing CD34\(^+\) and 1 of the following CPC markers: CD133, CXCR4, or VEGF2R. The authors confirmed the significance of renal insufficiency (estimated glomerular filtration rate [eGFR] < 60 ml/min/1.73 m\(^2\)) as an adverse predictor of cardiovascular outcomes, as it was associated with 48% increase in CV death/nonfatal MI and 39% increase in all-cause mortality. Of interest, the authors provide sufficient evidence that renal insufficiency was associated with an approximately 10% lower number of CPCs, a relationship that was independent of the presence of other cardiovascular risk factors in patients aged 70 years or older. Lower CPCs were associated with CV mortality in the whole study cohort as well as among patients with renal insufficiency.
insufficiency independent of eGFR, age, sex, race, traditional cardiovascular risk factors, hemoglobin, white blood cell count, or therapeutic regimen. Most importantly, the authors robustly demonstrated that addition of CPC number into a model including age, eGFR, current smoking, heart failure history, and hemoglobin level substantially improves the discriminative ability of the model for an adverse cardiovascular event (integrated discrimination index = 15% to 20%). Finally, the authors convincingly showed that patients with renal insufficiency and high CPC numbers had similar risks for CV death/MI with patients with normal renal function, whereas those with both renal insufficiency and low CPCs had 60% to 80% higher risks for CV death/MI and all-cause mortality. These results further confirm the importance of the interplay between chronic kidney disease (CKD) and CPC-mediated tissue repair in cardiovascular outcomes. Further research is warranted to delineate how CKD affects the number and function of PCPs and how PCPs support the maintenance of vascular health in patients with CKD.

This interesting study by Mehta et al. (2) sheds more light on the role of PCPs in renal insufficiency, confirming previous reports showing that low PCPs are associated with adverse cardiovascular events or cardiac remodeling post-MI (3). Endothelial progenitor cells have been previously associated with reduced risk of CV death, major cardiovascular events, or need for revascularization among patients with CAD after adjustment for age, sex, and traditional cardiovascular risk factors. In a recent meta-analysis encompassing 21 longitudinal studies on the prognostic role of PCPs for cardiovascular events, reduced CPC levels were associated with approximately 2-fold increased risk for future cardiovascular events or CV death as well as with increased risk of stent restenosis (4).

Recruitment of PCPs from bone marrow or peripheral niches to sites of tissue injury is a multi-step process requiring the mobilization, chemoattraction, rolling, and adhesion of PCPs onto vascular wall and migration into areas of tissue injury. We, and others, have previously shown that platelets have a central role in domiciliation and subsequent differentiation of PCPs. Platelets form aggregates with CD34+ PCPs, which adhere firmly to ischemic endothelium. The adhesion of platelet–CD34+ cell coaggregates onto the extracellular matrix and to endothelial monolayer is enhanced compared with CD34+ cells alone under high shear rates in vitro and within the microcirculation of mice after ischemia-reperfusion injury, as assessed by intravital microscopy (3). This CPC-platelet interaction is further enhanced by the platelet-derived chemokine stromal cell-derived factor-1 (SDF-1), which chemoattracts circulating CD34+ cells and enhances their adhesion to arterial endothelium (5). Adhesion of human CD34+ progenitor cells over platelets immobilized on the endothelium is further enhanced by JAM-A-JAM-A homophilic bonds and JAM-A-LFA-1 heterophilic bonds, which, along with binding of SDF-1 to its counter-receptor CXCR4, also drive the differentiation of CD34+ cells into endothelial progenitor cells (1,3,5). Circulating platelet-CD34+ cell coaggregates are significantly increased in acute MI, and their number was associated with a lower myocardial infarct size and better left-ventricular function 3 months post-MI (3). Whether CKD affects the formation of platelet-CD34+ cell coaggregates remains to be shown in future studies.

Multiple efforts have been made to capitalize on the regenerative capacity of endothelial progenitor cells in acute ischemic settings. Stents that capture endothelial progenitor cells have led to significant reduction of neointimal area and percent area stenosis in preclinical MI models, showing excellent safety and efficacy profiles, also in clinical trials. Moreover, administration of CD34+ cells reduced the risk for amputation in patients with critical limb ischemia, with the number of applied CD34+ cells being an independent predictor of limb salvage and wound healing. Similarly, administration of CD34+ cells was shown to improve pulmonary hemodynamics in pulmonary arterial hypertension. Despite promising early results, several issues regarding CD34+ availability and differentiation potential remain to be addressed; based on the number of cells used in animal models, several liters of blood would be required to isolate sufficient endothelial progenitor cells to treat critical limb ischemia in an average adult patient, which—in combination with the lower number of PCPs in patients with CAD and especially in those with renal insufficiency as shown in the current study—precludes the use of CD34+ cells in everyday clinical practice. Moreover, the differentiation potential and function of endothelial progenitor cells may be severely compromised in patients with CAD, and especially in those with renal insufficiency, because of increased senescence. Therefore, there is an imperative need for further basic and translational science studies addressing the
“rejuvenation” of CD34+ cells in ischemic and chronic kidney disease. Understanding how CKD affects CD34+ cells will pave the way toward the development of novel therapies, as this is a mechanism that, according to the convincing data provided in the current report by Mehta et al. (2), warrants further investigation.

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KEY WORDS CD34+ cells, circulating progenitor cells, endothelial progenitor cells, prognosis, renal insufficiency
Heart Failure in Humans Reduces Contractile Force in Myocardium From Both Ventricles

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HIGHLIGHTS

- Contractile assays were performed using multicellular preparations isolated from the left and right ventricles of organ donors and patients with heart failure.
- Heart failure reduced maximum force and power by approximately 30% in the myocardium from both ventricles.
- Heart failure increased the Ca\(^{2+}\) sensitivity of contraction, but the effect was bigger in right ventricular tissue than in left ventricular samples.
- The changes in Ca\(^{2+}\) sensitivity may reflect ventricle-specific post-translational modifications to sarcomeric proteins.

SUMMARY

This study measured how heart failure affects the contractile properties of the human myocardium from the left and right ventricles. The data showed that maximum force and maximum power were reduced by approximately 30% in multicellular preparations from both ventricles, possibly because of ventricular remodeling (e.g., cellular disarray and/or excess fibrosis). Heart failure increased the calcium (Ca\(^{2+}\)) sensitivity of contraction in both ventricles, but the effect was bigger in right ventricular samples. The changes in Ca\(^{2+}\) sensitivity were associated with ventricle-specific changes in the phosphorylation of troponin I, which indicated that adrenergic stimulation might induce different effects in the left and right ventricles.

Heart Failure Reduces Contractile Force

In healthy humans, the left ventricle (LV) is a thick-walled, bullet-shaped chamber that generates approximately 120 mm Hg during systole. Conversely, the right ventricle (RV) is a thinner crescent-shaped structure that produces approximately 25 mm Hg (1,2). Laplace’s law (3) shows that wall thickness will contribute to the systolic pressure difference, but it is not known whether interventricular differences in contractile function augment the structural effect.

The impact of heart failure on cells from the LV and RV is also unclear. Although numerous studies have shown that heart failure reduces maximum force and increases the calcium (Ca\(^{2+}\)) sensitivity of contraction of the permeabilized LV myocardium (4–10), few experiments have been performed using human RV samples. One recent study by Hsu et al. (11) demonstrated that the Ca\(^{2+}\) sensitivity of contraction was increased in the RV of patients who had systemic sclerosis-associated pulmonary arterial hypertension. However, these investigators were not able to study LV tissue. Previous experiments using rat models of heart failure have yielded apparently conflicting results. Belin et al. (12) demonstrated that heart failure reduced maximum force in myocytes from both ventricles. Conversely, Perreault et al. (13) showed that heart failure increased maximum force in RV tissue but had no effect on LV samples. Belin et al. (12) found that heart failure reduced Ca\(^{2+}\)-sensitivity in the LV without affecting the RV, whereas Perreault et al. (13) observed no change in LV samples but did observe an increase in the sensitivity of RV tissue.

This paper extends these studies to the human myocardium. The data showed that heart failure reduced the maximum force and power generated by multicellular preparations from both ventricles without changing contractile kinetics. Ca\(^{2+}\)-sensitivity was increased in failing myocardium from both ventricles, but the effect was greater for RV samples than that for LV tissue. Biochemical assays suggested that the sensitivity effects might reflect ventricle-specific modulation of the phosphorylation of cardiac troponin I (TnI).

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors’ institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the JACC: Basic to Translational Science author instructions page.

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TABLE 1 Summary of Clinical Characteristics and Medication Use for Patients and Organ Donors

| Percentage of patients/donors taking medication | Heart Transplant | Organ Donor | p Value |
|-----------------------------------------------|-----------------|-------------|---------|
| Age (yrs)                                      | 48.0 ± 17.3     | 41.7 ± 15.7 | 0.494   |
| Male                                          | 58.3            | 60.0        | 1.000   |
| Ejection fraction (%)                         | 24.3 ± 10.8     | 47.6 ± 15.6 | 0.006   |
| J--blocker                                     | 83.3            | 0.0         | 0.008   |
| ACE inhibitor                                  | 83.3            | 0.0         | 0.008   |
| Statin                                        | 66.7            | 0.0         | 0.234   |
| Aldosterone antagonist                         | 50.0            | 0.0         | 1.000   |
| Inotrope                                       | 83.3            | 0.0         | 0.008   |
| Digitalis                                      | 8.3             | 0.0         | 0.008   |
| Vasopressor                                    | 0.0             | 50.0        | 0.050   |
| Aspirin                                       | 75              | 25.0        | 0.118   |

Values are mean ± SD or %. p Values for age and ejection fraction were calculated using unpaired Student’s t-tests. All other p-values were calculated using Fisher’s exact tests. Data values that were unknown for a patient or organ donor were excluded from the analysis. ACE = angiotensin-converting enzyme.

METHODS

HUMAN SAMPLES. Myocardial samples were acquired at the University of Kentucky using a published protocol (14). Briefly, hearts from 12 patients who underwent cardiac transplantation (Supplemental Table S1) and from 5 organ donors who did not have heart failure (Supplemental Table S2) were handed to a researcher in the operating room, who then dissected samples from through-wall sections of the distal region of the LV and RV. LV samples were further dissected transmurally into sub-epicardial, mid-myocardial, and sub-endocardial specimens. RV samples were not separated transmurally because the RV wall is typically much thinner. Specimens were snap frozen in liquid nitrogen within approximately 30 min and stored in the vapor phase of liquid nitrogen until use. The University of Kentucky Institutional Review Board approved all procedures, and subjects or their legally authorized representatives gave informed consent.

Previous data from the laboratory (15) showed that heart failure had a greater impact on the contractile properties of the middle transmural region of the LV than on samples from the sub-epicardium or sub-endocardium. This study compared samples from the RV and the LV mid-myocardium to optimize the probability of detecting important effects.

Table 1 summarizes clinical characteristics and the use of medications for the patients and organ donors.

PREPARATIONS AND EXPERIMENTAL SETUP. Multicellular preparations were obtained by mechanical disruption of approximately 100 mg of tissue followed by chemical permeabilization (1% v/v Triton detergent). Preparations (860 ± 248 µm) from 17 patients (5 nonfailing and 12 failing hearts) were attached between a force transducer (resonant frequency, 600 Hz; Model 403, Aurora Scientific, Aurora, Ontario, Canada) and a motor (step time 0.6 ms; model 312B, Aurora Scientific) and stretched to a sarcomere length of 2.24 ± 0.01 µm in pCa (−log10[Ca2+]) 9.0 solution. The cross-sectional area (4.05 ± 2.08 × 10−8 m²) was estimated from the width of the preparation, assuming a circular profile. Measurements were performed at 15 °C using SLControl software (16).

FORCE AND Ca²⁺ SENSITIVITY MEASUREMENTS. As described by Haynes et al. (15), preparations were initially activated in a solution with a saturating Ca²⁺ concentration of pCa 4.5. Maximum force was defined as the steady-state force measured during this trial. Subsequent trials measured force in solutions with higher pCa values. Force values were corrected for potential preparation-dependent run down using reference contractions measured in pCa 9.0 and pCa 4.5 solutions throughout the protocol.

Tension pCa curves were calculated by fitting a function of the form:

$$F = F_{\text{pass}} + F_{\text{act}} \left[ \frac{[Ca^{2+}]_{\text{Hi}}}{[Ca^{2+}]_{\text{Hi}}} + [Ca^{2+}]_{\text{Hi}} \right]$$

(Eq. 1)

to the data, where $F_{\text{pass}}$ is the passive force, $F_{\text{act}}$ is maximum Ca²⁺-activated force, $n_H$ is the Hill coefficient, and is the free Ca²⁺ concentration needed to develop half-maximum Ca²⁺-dependent force. Thirty-one of the 98 preparations studied were discarded from the analysis because the force measured in pCa 4.5 solution dropped by >30% during the protocol (n = 19), or the r² value for the fit to the Hill curve was <0.95 (n = 12).

POWER MEASUREMENTS. Myocardial power output was measured by allowing each preparation to shorten against pre-set loads for 80 ms in pCa 4.5 solution. Shortening velocity was calculated as the rate of change of fiber length during the final 50 ms of each force clamp. A hyperbolic equation of the form $(F+a) (V+b) = (F_{\max}+a) b$ was then fitted to the dataset, where $F$ is force, $F_{\max}$ is the isometric force, and $a$ and $b$ are constants. $V_{\max}$, the preparation’s maximum shortening velocity, was estimated by extrapolating the equation to zero force.
Because power is the product of force and velocity, power-force curves were calculated by fitting the following function:

\[ P = F \cdot b \left( \frac{F_{\text{max}}}{F + a} - 1 \right) \]  
(Eq. 2)

for the experimental data. Maximum power, \( P_{\text{max}} \), was calculated as the greatest value of Equation 2. Data from the 43 preparations, in which the r2 value for the power-force fit was <0.9, were analyzed.

**BIOCHEMICAL ASSAYS.** The phosphorylation statuses of TnI, myosin binding protein-C (MyBP-C), and myosin regulatory light chain (RLC) were analyzed using gel electrophoresis as described by Haynes et al. (15). The samples used in these gels were saved from the homogenate after the mechanical disruption and chemical permeabilization procedures were used to prepare samples for contractile assays. Phosphorylated proteins were assessed using Pro-Q Diamond (Invitrogen, Carlsbad, California). Total protein was measured using SYPRO Ruby (Invitrogen). Data from multiple gels were collated and normalized to values from a single reference control that was included in every experiment.

TnI Ser23/24 specific phosphorylation was determined by Western blot as described by Nixon et al. (17) and Salhi et al. (18) using the following antibodies: rabbit anti-phosphorylated TnI Ser-23/24 (Cell Signaling Technology, Inc., Danvers, Massachusetts), anti-rabbit Cy5-labeled secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pennsylvania), mouse anti-cardiac TnI antibody (clone 5; Fitzgerald, Acton, Massachusetts), and anti-mouse Cy2 labeled fluorescent secondary antibody (Jackson ImmunoResearch Laboratories).

**STATISTICAL ANALYSIS.** Data were analyzed in SAS version 9.4 (SAS Institute, Inc., Cary, North Carolina) using linear mixed effects models that incorporated 2 main effects (heart failure status and ventricular region) and their interaction. Testing for the interaction determined whether the effect of 1 independent variable depended on the other. An example of an interaction is a value that decreases with heart failure in samples from the LV but increases with heart failure in samples from the RV, so that the effect of heart failure depends on the ventricle that is being studied.

As previously described (15), the linear mixed model accounted for the hierarchical nested structure of the data (values obtained from multiple preparations from different regions of the same hearts) and had greater statistical power than a standard 2-way analysis of variance test for this type of experimental design. Post hoc analyses were performed using Tukey-Kramer corrections that took into account the number of hearts and the number of samples from each heart. The p values <0.05 were considered significant. Data are reported as mean ± SEM, unless otherwise specified. Data could be clinically significant although statistical significance was not achieved, especially with smaller sample sizes.

**RESULTS**

**HEART FAILURE REDUCES \( \text{Ca}^{2+} \)-ACTIVATED FORCE IN MULTICELLULAR PREPARATIONS FROM BOTH VENTRICLES.** Figure 1 and Supplemental Figure 1 show force normalized to the cross-sectional area as a function of the activating \( \text{Ca}^{2+} \) concentration (pCa) for multicellular preparations from the LVs and RVs of organ donors and patients with heart failure. The solid lines are the best-fits of Equation 1 to the mean data values measured at each \( \text{Ca}^{2+} \) concentration for each type of preparation.

Figure 2 shows passive forces (\( F_{\text{pas}} \)) and \( \text{Ca}^{2+} \).activated forces (\( F_{\text{act}} \)) calculated by fitting Equation 1 to the data for each experimental preparation. The statistical analysis showed that \( F_{\text{pas}} \) did not depend on heart failure status or ventricular region. In contrast, \( F_{\text{act}} \) was reduced by >30% in samples from failing hearts (p < 0.001). Similar reductions were observed in the samples from the LVs and RVs (i.e., there was no effect of cardiac region).

**HEART FAILURE HAS A GREATER EFFECT ON THE \( \text{Ca}^{2+} \) SENSITIVITY OF THE RV.** Figure 3 shows the \( \text{Ca}^{2+} \) sensitivity (pCa50) and the cooperativity (nH) calculated for each multicellular preparation. The pCa50 values were higher for the failing samples than that for preparations from the organ donors (p = 0.005) but there was a statistically significant interaction between disease status and cardiac region (p = 0.029). The interaction meant that the increase in calcium sensitivity induced by heart failure was statistically greater in samples from the RV than in samples from the LV. In addition, the post hoc tests showed that in failing hearts, samples from the RV were more sensitive to \( \text{Ca}^{2+} \) than the samples from the LV (p = 0.039). No statistically significant effects were observed for the nH values.

**HEART FAILURE REDUCES THE POWER OUTPUT OF MYOCARDIUM FROM BOTH VENTRICLES.** Power quantified the rate at which the myocardial preparations performed mechanical work and was calculated as the product of force and velocity. Figure 4A to 4C illustrates the experimental technique that was used to calculate the maximum shortening velocity (Vmax) and the maximum power output (Pmax) of a single representative preparation. Data from multiple
preparations are shown in Figure 4D and 4E. As in the case of $F_{\text{act}}$ (Figure 2B), $P_{\text{max}}$ was reduced by approximately 30% in samples from both the LVs and RVs of failing hearts. In contrast, no statistically significant effects were observed for $V_{\text{max}}$. $V_{\text{max}}$ is believed to depend on cross bridge cycling kinetics (19), which can be estimated by measuring the rate of force recovery ($k_{tr}$) after a quick shortening and/or re-stretch perturbation. Figure 5A shows representative records measured for a LV preparation isolated from an organ donor. Consistent with previous results from human myocardium (15), force overshot the steady-state isometric value during the recovery phase (20,21). The $k_{tr}$ values increased approximately linearly with steady-state isometric force in every experimental group (Figure 5B). The rates measured in maximally activating pCa 4.5 solution did not depend on heart failure status or ventricular region.

Data for $k_{tr}$ values measured at sub-maximal activation levels ranging from pCa 6.0 to pCa 5.2 are shown in Supplemental Figure S2. The kinetics were faster in the samples from patients with heart failure at some activation levels, with the difference being more pronounced in the RV. The physiological significance of these results is not yet clear.

HEART FAILURE MODULATES THE PHOSPHORYLATION OF MYOFILAMENT PROTEINS. Figure 6A shows images from a representative gel that was stained with Pro-Q Diamond to show phosphorylated proteins and then SYPRO Ruby to show total protein. Figure 6B to 6D shows data obtained from similar images quantifying the phosphorylation levels of TnI, MyBP-C, and RLC. Both TnI and RLC exhibit a statistical interaction ($p = 0.037$) between heart failure status and the cardiac region with the phosphorylation levels trending higher in RV samples from organ donors and lower in RV samples from patients with heart failure.

The data for MyBP-C showed a different pattern. No interaction was observed, but there was a main effect of heart failure status ($p = 0.010$). This implied that the phosphorylation of MyBP-C was lower in samples from both ventricles in failing hearts.

Supplemental Figures S3 to S7 show the relative phosphorylation of TnI, MyBP-C, and RLC plotted against pCa50, nH, Fpas, Fact, and the maximum value of $k_{tr}$. Deming regression (which takes into account experimental uncertainty in both the x and y coordinates) showed that pCa50 was negatively correlated with the relative phosphorylation of both MyBP-C and RLC, and that Fact was positively correlated with the relative phosphorylation of MyBP-C.

Pro-Q Diamond is commonly used to study sarcomeric proteins (22–24) but the stain is not completely specific to phosphorylated proteins and

**FIGURE 1 Force Pca Curves**

| Region | pCa50 | nH |
|--------|-------|----|
| LV     | 5.62 ± 0.05 | 2.90 ± 0.32 |
| RV     | 5.53 ± 0.03 | 2.19 ± 0.35 |

| Region | pCa50 | nH |
|--------|-------|----|
| LV     | 5.67 ± 0.01 | 2.67 ± 0.17 |
| RV     | 5.73 ± 0.02 | 2.84 ± 0.13 |

Data for multicellular preparations isolated from the left ventricle (LV) and right ventricle (RV) of (A) organ donors and (B) patients who had heart failure. Symbols and table entries show mean ± SEM. Data were acquired as follows: LV organ donor, 9 preparations from 4 hearts; RV organ donor, 12 preparations from 5 hearts; LV heart failure, 22 preparations from 10 hearts; RV heart failure, 24 preparations from 11 hearts. $pCa = -\log_{10}[Ca^{2+}]$. nH = Hill coefficient.
Western blotting (Figure 7) was performed to address these potential limitations. As for the experiments performed using Pro-Q Diamond (Figure 6B), the phosphorylation status of TnI 23/24 exhibited a statistical interaction (p = 0.039) between heart failure status and cardiac region, which indicated that the did not provide information about post-translational modifications to identified residues. Western blotting (Figure 7) was performed to address these potential limitations. As for the experiments performed using Pro-Q Diamond (Figure 6B), the phosphorylation status of TnI 23/24 exhibited a statistical interaction (p = 0.039) between heart failure status and cardiac region, which indicated that the
effect of heart failure on TnI phosphorylation depends on the ventricle.

**DISCUSSION**

To our knowledge, this is the first study that systematically investigated how heart failure in humans affects the contractile properties of chemically permeabilized myocardial samples from LVs and RVs. The main findings are that heart failure: 1) reduces isometric force and maximum power output in preparations from both ventricles; and 2) increases the calcium sensitivity (pCa50) of RV tissue more than that of LV tissue. Biochemical data suggested that the changes in calcium sensitivity might reflect ventricle-specific modulation of TnI phosphorylation.

**REDUCED ISOMETRIC FORCE AND POWER IN FAILING TISSUE MAY REFLECT VENTRICULAR REMODELING.** Figures 1 and 2B show that heart failure reduced maximum Ca^{2+}-activated force by at least 30% in multicellular preparations from both ventricles. Maximum power was also reduced by a similar amount in both ventricles (Figure 4D). In principle, these effects could reflect a change in the fractional volume occupied by sarcomeres and/or alterations to cross-bridge kinetics that reduce the force-generating capacity of myosin heads in failing ventricles.
Figures 4E, 5B, and 5C show that neither Vmax nor ktr were affected by heart failure status or cardiac region at maximal levels of Ca$^{2+}$ activation. Both parameters are indicative of cross-bridge kinetics and would be expected to change if the attachment and/or detachment rates of myosin were altered. Thus, the present data did not support a kinetic basis for the reduction in force. Instead, the mostly likely explanation for the approximate 30% reduction in force and power in failing samples was a decrease in the fractional volume occupied by contractile machinery.

This conclusion was consistent with a previous study by Haynes et al. (15) that compared the contractile properties of multicellular preparations from the sub-endocardial, mid-myocardial, and sub-epicardial regions of the LV free wall of organ donors and patients with heart failure. Histological data (staining with picrosirius red) included in that study showed that the collagen to tissue ratio fell from approximately 27% in mid-myocardial tissue from patients with heart failure to approximately 15% in corresponding samples from organ donors. Unfortunately, histological data from RV samples were not available for the present study. However, if the preparations had exhibited similar trends to those reported by Haynes et al., the increased fibrosis in the failing samples could account for approximately 50% of the reductions shown in Figures 1, 2B, and 4E. Other potential mechanisms that could reduce maximum contractile force in failing myocardium include disarray of myocytes and/or myofibrils (25) and changes in the fractional volume occupied by mitochondria (26). These possibilities could be tested in future work: 1) measuring the contractile function of single myocytes that do not have extracellular...
collagen (27); and 2) analyzing tissue samples with immunohistochemistry and electron microscopy (28).

**INTERVENTRICULAR DIFFERENCES IN Ca^{2+} SENSITIVITY ARE ASSOCIATED WITH PHOSPHORYLATION OF TnI.** Because myocardium is only transiently activated during each heart beat, its sensitivity to Ca^{2+} (quantified as a pCa_{50} value) is critically important for function. For example, all other things being equal, myocytes with higher pCa_{50} values will develop more contractile force in response...
to a given intracellular Ca\textsuperscript{2+} transient than myocytes with low Ca\textsuperscript{2+} sensitivities. Figure 3A confirmed previous work (7,29) that showed that myocardium from failing hearts is more sensitive to Ca\textsuperscript{2+} than tissue from organ donors (p < 0.005). One possibility is that the failing heart enhances Ca\textsuperscript{2+} sensitivity to try and compensate for its reduced force-generating capacity. Supplemental Figure S1 superposes force pCa curves measured with samples from organ donors and patients with heart failure. The curves nearly overlap at the sub-maximal activation levels where the heart normally operates.

The present data also revealed a new statistical interaction between heart failure status and cardiac ventricles. More specifically, heart failure increased the Ca\textsuperscript{2+} sensitivity of RV myocardium more than that of LV tissue. Figures 6 and 7 show data that provide insights into the molecular mechanisms that drive these effects. Two independent measures of TnI phosphorylation (Pro-Q Diamond staining [Figure 6B] and Western blotting with an antibody specific to Ser-23/24 phosphorylation [Figure 7B]) showed a statistical interaction between disease status and the cardiac ventricle. It is well known that phosphorylation of TnI (most notably at serine 23/24) reduces Ca\textsuperscript{2+} sensitivity (4,6,10,30–35); therefore, these statistical interactions are consistent with post-translational modifications to TnI contributing to the ventricle-specific effects.

Phosphorylation of RLC also exhibited a significant interaction (Figure 6C) with higher phosphorylation levels again being associated with reduced Ca\textsuperscript{2+} sensitivity. This effect is also evident in Supplemental Figure S3C, which shows the relative phosphorylation of RLC plotted against pCa50. This was a more complex relationship because increased phosphorylation of RLC was shown to enhance Ca\textsuperscript{2+} sensitivity (36,37). One possibility was that the phosphorylation of RLC changes during human heart failure partially compensated for the post-translational modifications to TnI and mitigated diastolic dysfunction. A similar conclusion was
reached by van der Velden et al. (5), who observed changes in the phosphorylation of TnI and RLC in human heart failure that were similar to those shown here.

Figure 6D shows that MyBP-C was dephosphorylated in failing samples from both ventricles. This modification increased myofilament Ca\(^{2+}\) sensitivity (38-41) and could therefore be driving the global increase in Ca\(^{2+}\) sensitivity observed in the failing samples (Figure 3A and Supplemental Figure S3B).

The physiological basis of the interventricular difference in Ca\(^{2+}\)-sensitivity remains unclear. One possibility is that the ventricles are tuned to different mechanical loads. Another possibility is that they respond differently to \(\beta\)-adrenergic stimulation. Recent work that used Förster Resonance Energy Transfer (FRET) biosensors suggested that maximal inotropy required compartmentalized signaling responses (42). In addition, Surdo et al. (43) reported an enrichment in the expression of genes involved in adrenergic signaling in calls from the RV of healthy human donors in comparison to cells from the LV using single cell RNA-Seq. Building on this theme, Molina et al. (44) showed that in healthy dogs, the RV was more sensitive to \(\beta\)-adrenergic stimulation and exhibited increased activity of protein kinase A (PKA). This enzyme phosphorylates TnI (45) so differential responses to \(\beta\)-adrenergic stimulation could contribute to the effects shown in Figures 6B and 7B. Interestingly, Hsu et al. (11) showed that treatment with PKA equalized the Ca\(^{2+}\) sensitivity of RV myocardium from organ donors and in patients with systemic sclerosis-associated pulmonary hypertension, a finding that further reinforced the importance of PKA signaling in disease. However, the present data suggested that PKA was unlikely to be the only mechanism that contributed to interventricular differences because enhanced activity of the kinase in the RV should also have increased the phosphorylation of MyBP-C. This was not observed (Figure 6C).

STUDY LIMITATIONS. Although experiments using human biospecimens sometimes have greater translational significance than studies that only use animals, they also have limitations. For example, it was exceedingly difficult to control for all of the potential confounders (including age, sex, genetic background, and comorbidities) that could have influenced the data (46). Some clinical data were also unavailable. This reduced the statistical power of tests that investigated potential relationships between cellular and organ-level function and was a particular concern for parameters related to the RV (Supplemental Table S3).

Sample processing was another concern. All of the hearts used were procured by the same personnel and cooled in ice cold saline within moments of being removed from the patient or donor (14). Nevertheless, the mean time from procurement to the sample being frozen in liquid nitrogen was approximately 30 min. As demonstrated by Walker et al. (47), this is clearly enough time to alter the post-translational status of sarcomeric proteins, which sometimes changes on a beat-to-beat basis (48).

Another issue was that the patients and organ donors were treated with drugs that were intended to alter contractile properties. Catecholamine levels also rose during tissue procurement (46), which might have added additional complications. Again, it was difficult to know how to overcome these limitations in a translational research setting without having a negative impact on patient care.

Finally, although this study investigated the phosphorylation of several key myofilament proteins, the experiments could have missed other critical modifications. This could be addressed in future work using an unbiased approach (e.g., phosphoproteomics).

CONCLUSIONS

This study presented 3 important results. First, heart failure in humans depressed maximum contractile force and maximum power by similar amounts in myocardium from both ventricles. Second, human heart failure increased the Ca\(^{2+}\) sensitivity of RV myocardium more than the Ca\(^{2+}\) sensitivity of LV myocardium. Third, the differences in Ca\(^{2+}\) sensitivity were likely to involve interventricular differences because enhanced activity of the kinase in the RV should also have increased the phosphorylation of MyBP-C. This was not observed (Figure 6C).

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ADDRESS FOR CORRESPONDENCE: Dr. Kenneth S. Campbell, Department of Physiology, University of Kentucky, Medical Science Building MS-508, 800 Rose Street, Lexington, Kentucky 40536-0298. E-mail: k.s.campbell@uky.edu.
COMPETENCY IN MEDICAL KNOWLEDGE: This study showed that heart failure in humans reduced the force and power generated by myocardium from the LVs and RVs. The kinetics of the contraction (assessed as shortening velocity and the rate of force generation) were not markedly affected, so the contractile dysfunction was most likely to reflect remodeling (e.g., excess fibrosis and/or cellular disarray). In addition, heart failure increased the Ca\(^{2+}\) sensitivity of contraction, with the effect being greater in RV samples. These changes were associated with altered phosphorylation of TnI and MYP-C, which indicated that adrenergic signaling might induce different consequences in the LVs and RVs.

TRANSLATIONAL OUTLOOK: Although technically challenging, more studies are required to determine how the Ca\(^{2+}\) sensitivity of contraction varies in different regions of failing hearts. Abnormalities in these patterns might contribute to regional hypokinesia and/or hyperkinesia.

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KEY WORDS Ca^{2+} sensitivity, heart failure, human myocardium, myofilament proteins, ventricular function

APPENDIX For supplemental figures and tables, please see the online version of this paper.
Optogenetic Stimulation of Vagal Efferent Activity Preserves Left Ventricular Function in Experimental Heart Failure

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VISUAL ABSTRACT

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Optogenetic Vagus Nerve Stimulation Preserves LV Function

C hronic heart failure is 1 of the most common causes of morbidity and mortality in the developed world. Established pharmacological treatment of heart failure that involves several drug classes, including β-blockers and inhibitors of renin-angiotensin-aldosterone system, improves symptoms and reduces mortality. Yet, drug therapy remains insufficient because cardiac function continues to deteriorate over time, and most patients have poor prognosis [1].

Autonomic dysfunction characterized by sympathetic activation and parasympathetic (vagal) withdrawal accelerates the development and progression of the disease [2-4]. The current gold standard pharmacological treatment includes drugs that limit the sympathetic effects on the heart and kidneys (e.g., β-blockers). However, stimulation of vagal efferent activity to increase parasympathetic tone and redress autonomic balance as a treatment for heart failure has proved to be more difficult to achieve [5,6]. Patients with heart failure on optimal medical therapy with persistent autonomic dysfunction have the worst prognosis [7].

Electrical vagus nerve stimulation (VNS) has been shown to reduce the extent of an acute myocardial infarction (MI) [8-11] and slow the progression of myocardial remodeling, as well as atrial and ventricular dysfunction in animal models of chronic heart failure [12-17]. In the first human trial that involved 32 patients with heart failure, De Ferrari et al. [18] demonstrated that electrical stimulation of the right vagus nerve using implanted devices improved New York Heart Association functional class, left ventricular (LV) function, and quality of life. However, subsequent multicenter trials designed to test VNS efficacy in large cohorts of patients with heart failure reported mixed results. Premchand et al. [19] showed improvements of the ejection fraction and symptoms in another noncontrolled study that involved 60 patients (ANTHEM-HF [Autonomic Neural Regulation Therapy to Enhance Myocardial Function in Heart Failure] study),

MANUSCRIPT

HIGHLIGHTS

- This study was designed to determine the effect of selective optogenetic simulation of vagal efferent activity on left ventricular function in an animal (rat) model of MI-induced heart failure.
- Optogenetic stimulation of dorsal brainstem vagal pre-ganglionic neurons transduced to express light-sensitive channels preserved LV function and exercise capacity in animals with MI.
- The data suggest that activation of vagal efferents is critically important to deliver the therapeutic benefit of VNS in chronic heart failure.

SUMMARY

Large clinical trials designed to test the efficacy of vagus nerve stimulation (VNS) in patients with heart failure did not demonstrate benefits with respect to the primary endpoints. The nonselective nature of VNS may account for the failure to translate promising results of preclinical and earlier clinical studies. This study showed that optogenetic stimulation of vagal pre-ganglionic neurons transduced to express light-sensitive channels preserved left ventricular function and exercise capacity in a rat model of myocardial infarction–induced heart failure. These data suggested that stimulation of vagal efferent activity is critically important to deliver the therapeutic benefit of VNS in heart failure. (J Am Coll Cardiol Basic Trans Science 2020;5:799–810)

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whereas Zannad et al. (20) and Gold et al. (21) reported no effect of VNS on LV remodeling and function or mortality in trials that involved 86 (NECTAR-HF [Neural Cardiac Therapy for Heart Failure]) and 707 (INOVATE-HF [Increase of Vagal Tone in Chronic Heart Failure]) patients. However, there were reported improvements in quality of life (20).

The mechanisms underlying the potential benefit of VNS in heart failure remain poorly understood. The cervical vagus is a mixed nerve containing sensory and motor fibers at an approximate ratio of 4:1. The nerve bundle contains branches that innervate most of the viscera and sparse sympathetic fibers. There is no experimental data to suggest that the VNS beneficial effects on the failing heart observed in animal and some human studies are due to the recruitment of afferent (sensory) or efferent (motor) vagal fibers (or both) by the electrical current pulses applied to the nerve at the cervical level. Although attempts had been made to design VNS devices for preferential stimulation of vagal efferents (5,18), electrical properties of the nerve fibers that constitute the human vagus nerve make selective fiber recruitment virtually impossible to achieve.

Further refinement of VNS as a potential treatment for human heart failure may require development of alternative methods of selective stimulation of specific groups or subsets of vagal fibers. Selective stimulation would be expected to maximize the efficacy and limit the side effects associated with the recruitment of fibers that may not necessarily confer any benefit on the failing heart (22). The optogenetic approach involving transduction of mammalian neurons to express the native or modified light-sensitive channels from algae and activation of these neurons using light provides the required level of selectivity for experimental studies (23). Recently, we and other laboratories used optogenetic techniques to study the nervous mechanisms controlling the heart (11,24,25). Our research group demonstrated that stimulation of approximately 300 to 400 vagal pre-ganglionic neurons in the dorsal motor nucleus of the vagus nerve (DVMN) markedly reduced the extent of MI in an animal model (11). These data provided direct evidence of effective cardioprotection by selective stimulation of vagal efferent activity. In the present study, we genetically targeted the DVMN neurons to express the light-sensitive channel ChIEF and determined the effect of selective stimulation of the DVMN vagal efferent projections using light (optoVNS) on LV function in a rat model of MI-induced heart failure.

METHODS

All the experiments were performed in accordance with the European Commission Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the United Kingdom Home Office (Scientific Procedures) Act (1986) with project approval from the University College London Institutional Animal Care and Use Committee. The rats were group housed and maintained on a 12-h light cycle (lights on 0700) and had ad libitum access to water and food.

GENETIC TARGETING OF THE DVMN VAGAL PRE-GANGLIONIC NEURONS. Vagal pre-ganglionic neurons of the DVMN express transcriptional factor Phox2 and were targeted to express a light-sensitive chimeric channelrhodopsin derivative ChIEF fused with a fluorescent protein tdTomato (ChIEF-tdTomato) or enhanced green fluorescent protein (eGFP) (control transgene) using lentiviral vectors (LVV). Transgene expression was driven under the control of a Phox2-activated promoter PRSx8 (26).

Validation of the specificity of vectors in transducing the DVMN neurons was described in detail previously (11,27). Pulses of blue (445 nm) light trigger precisely timed depolarizations and action potential firing of the DVMN neurons transduced to express ChIEF (11).

Male Sprague-Dawley rats (150 to 200 g; n = 68) were anesthetized with a combination of ketamine (60 mg/kg intramuscularly) and medetomidine (250 µg/kg intramuscularly). Adequate depth of surgical anesthesia was maintained and confirmed by the absence of a withdrawal response to a paw pinch. With the head of the animal in a stereotaxic frame, a midline dorsal neck incision was made to expose the dorsal brainstem surface. Caudal populations of the DVMN neurons were targeted bilaterally with 1 microinjection on each side that delivered viral particles of either LVV-PRSx8-eGFP or LVV-PRSx8-ChIEFtdTomato (Figure 1A). The microinjections (0.5 µl at a rate of 0.1 µl/min) were made at 0.5 mm rostral, 0.6 mm lateral, and 0.8 mm ventral from the calamus scriptorius. After the microinjections, the wound was sutured, and anesthesia was reversed with atipamezole (1 mg/kg; intramuscularly). Postoperatively, the animals received buprenorphine (0.05 mg/kg/day, subcutaneously) analgesia for 3 days. No complications were observed after the surgery, and the animals gained weight normally.

OPTRODE IMPLANTATION. Two weeks after the microinjections of viral vectors, the animals were
anesthetized with ketamine (60 mg/kg intramuscularly) and medetomidine (250 μg/kg intramuscularly), and an optrode (Art Photonics, Berlin, Germany) was implanted to reach the dorsal surface of the brainstem for optimal light delivery to the groups of transduced vagal neurons. Two screws were placed into the skull, and the implant was secured in place with dental acrylic (Figure 1A). Anesthesia was...
reversed with atipamezole (1 mg/kg intramuscularly). Carprofen (5 mg/kg/day subcutaneously) was given for post-operative analgesia for 3 days, and the animals were allowed to recover for 7 days. Rats were 300 to 350 g at the time of the main experiment.

RECORDING THE EFFERENT ACTIVITY OF THE VAGUS NERVE. Rats (n = 4) transduced to express ChiEFtdTomato by the DVMN neurons were anesthetized with urethane (induction: 1.3g/kg, intraperitoneally; maintenance: 10 to 25 mg/kg/h intravenously) and instrumented as described in detail previously (28,29). Adequate anesthesia was ensured by maintaining stable levels of arterial blood pressure (ABP) and heart rate and showing lack of responses to a paw pinch. The femoral artery and vein were cannulated for measurements of blood pressure and administration of anesthetic, respectively. The trachea was cannulated, and the animal was mechanically ventilated with air supplemented with oxygen using a positive pressure ventilator with a tidal volume of approximately 1ml/100g of body weight and ventilator frequency similar to the resting respiratory rate (~60 strokes/min). Partial pressure of oxygen, partial pressure of carbon dioxide, and pH of arterial blood were measured regularly and kept within physiological ranges (partial pressure of oxygen: 95 to 105 mm Hg; partial pressure of carbon dioxide: 35 to 45 mm Hg, and pH 7.35 to 7.45) by adjusting the tidal volume and/or ventilator frequency as well as the level of supplemental oxygen. Body temperature was maintained at 37.0 ± 0.5°C. The right vagus nerve was dissected, isolated from the surrounding tissues, placed on silver wire recording electrodes, and covered with dental impression material. The recorded signal was amplified (~10,000), filtered (80 to 1,500 Hz), and sampled at a rate of 5 kHz using a Power1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, United Kingdom).

MODEL OF MI-INDUCED HEART FAILURE. MI leading to progressive impairment of LV function in rats was induced using a left anterior descending coronary artery (LAD) occlusion technique described in detail in the published reports (30). One week following optrode implantation (Figure 1A), the rats were anesthetized with ketamine (60 mg/kg intramuscularly) and medetomidine (250 μg/kg intramuscularly), and artifically ventilated following endotracheal intubation. A left thoracotomy was performed to expose the heart, the pericardium was opened, the heart was exteriorized, and the LAD was ligated just below the left atrial appendage using 4-0 braided silk suture. Successful coronary occlusion was confirmed by pallor of the anterior LV wall. Sham ligations involved the same sequence of procedures, including placement of the suture through the ventricular wall but without the occlusive tying. The chest incision was closed, the lungs were re-inflated, and artificial ventilation was discontinued once the animal started to breathe spontaneously. Anesthesia was reversed with atipamezole (1 mg/kg intramuscularly). Carprofen (5 mg/kg/day subcutaneously for 3 days) was given for post-operative analgesia. Post-operative mortality within the first 48 h after MI was 26%. All animals that survived beyond 48 h post-MI also survived the remainder of the study period.

EXPERIMENTAL GROUPS. On the basis that the minimum physiologically relevant effect of efferent vagus stimulation would be to prevent reduction in the ejection fraction after LAD occlusion by 8%, we estimated that at least 8 animals in each of the 4 experimental groups would be required (α = 0.01; 1-β = 0.9). The rats were randomized into 4 experimental groups (the rationale for the number of animals included in each of the groups at the beginning of the study is given in the following): 1) sham/shamVNS (n = 8): sham-operated animals expressing eGFP in the DVMN; 2) sham/optoVNS (n = 10): sham-operated animals expressing ChiEFtdTomato in the DVMN; 3) post-MI/shamVNS (n = 13): animals with LAD occlusion expressing eGFP in the DVMN; and 4) post-MI/optoVNS (n = 19): animals with LAD occlusion expressing ChiEFtdTomato in the DVMN (Figure 1A). Ten animals were included in sham/optoVNS group to allow for 20% dropout rate due to a potential lack of (or weak) ChiEFtdTomato expression in the DVMN due to variations of titer between different preparations of the vector. Thirteen animals were included in post-MI/shamVNS group to allow for an approximate 40% dropout of subjects that developed small infarcts (~30%). Nineteen rats were included in post-MI/optoVNS to allow for a potential 20% dropout rate due to lack of ChiEFtdTomato expression and/or 40% dropout due to the development of small infarcts.

The dorsal brainstem was illuminated in all the animals via the implanted optrode with 445-nm light pulses (10 ms, 15 Hz) for 15 min every 48 h for 4 weeks, which commenced 48 h after the LAD occlusion or sham surgery (Figure 1A). To minimize the stress of the animals, light stimulation was performed under mild sedation (1% isoflurane in 1:1 oxygen/air mixture).
EVALUATION OF EXERCISE CAPACITY. The exercise capacity of experimental rats was assessed using a single lane treadmill (Panlab Harvard Apparatus, Barcelona, Spain) as previously described (24,31). To determine the exercise capacity, treadmill speed was raised in increments of 5 cm/s every 5 min after an initial 15 min at 20 cm/s until the animal was unable to maintain pace with the moving belt and entered the stationary platform at the end of the treadmill 4 times within a 2-min period, which was the humanely defined point of exhaustion. The distance covered by the animal was recorded, and exercise capacity expressed as work done in Joules.

ECHOCARDIOGRAPHY. The animals were anesthetized with isoflurane (induction 4%, maintenance 2% in 1:1 oxygen/air mixture). Echocardiographic assessment of LV function was performed using a Vevo 2100 high-resolution ultrasound system with a MS250 13-24MHz linear array transducer (VisualSonics, Toronto, Ontario, Canada), with simultaneous heart rate monitoring (lead II electrocardiography). In a B-mode acquisition, the ejection fraction was determined using a parasternal long-axis view of the LV, measuring the length of the ventricle at the end of systole and diastole. Three perpendicular short-axis images were acquired equidistantly along the length of the LV to segment the endocardial border. The LV volumes were approximated using Simpson's rule (32). Using an apical 4-chamber view in Doppler mode, blood flow velocity across the mitral valve was also measured. The ratio of flow velocities during the early and atrial ventricular filling phases (E/A ratio) was calculated to assess diastolic function (33).

HEMODYNAMIC STUDIES. After the echocardiographic study, all animals underwent invasive hemodynamic assessment under urethane (1.3 g/kg intraperitoneally) anesthesia. Adequate depth of anesthesia was ensured by stable levels of ABP and heart rate in addition to the absence of a withdrawal response to a paw pinch. The femoral artery was cannulated, and a sequence of transverse slices (30,34,35). Briefly, the LV was cut into 5 to 6 transverse slices of approximately 1.5 mm thickness. The images of the slices were taken, and the length of the entire endocardial and epicardial circumferences, as well as the length of the scar muscle for endocardial and epicardial surfaces, were determined by computerized planimetry using ImageJ software (National Institutes of Health, Bethesda, Maryland). The ratio of the length of the scar and the entire surface circumferences defined the infarct size for endocardial and epicardial surfaces. Infarct size (percentage) was calculated as an average of the infarcted endocardial and epicardial surfaces. The extent of myocardial necrosis of these well-healed infarcts was determined from the circumference rather than from the volume of the infarct because the latter underestimates the initial loss of the myocardium (30).

HISTOLOGY. At the end of the hemodynamic study, the brains were removed and fixed in 4% phosphate-buffered (0.1 M, pH 7.4) paraformaldehyde solution. After cryoprotection (30% sucrose), the brainstem was isolated, and a sequence of transverse slices (30 μm) was cut to determine the extent of ChiEFtdTomato or eGFP expression by the DVMN neurons.

DATA ANALYSIS. Recordings of the vagus nerve activity and cardiovascular variables were analyzed using Spike2 software. Differences between the experimental groups were assessed using GraphPad Prism 6 software (GraphPad, San Diego, California). Comparisons were made using 2-way analysis of variance followed by Sidak’s p value correction for multiple comparisons or Student’s t-test, as appropriate. Data were reported as individual values and mean ± SEM. Differences with p < 0.05 were considered significant.
RESULTS

Expression of ChIEFtdTomato by the vagal pre-ganglionic neurons residing in the caudal regions of the left and right DVMN was confirmed in all the animals that received bilateral microinjections of LVV-PRSx8-ChIEFtdTomato (Figures 1B and 1C). Strong tdTomato fluorescence was observed in the ventral projections of the transduced DVMN neurons, forming the efferent vagus nerve (Figure 1B). Pulses of 445 nm light (10 ms) applied to the dorsal brainstem of rats transduced to express ChIEFtdTomato by the DVMN vagal pre-ganglionic neurons triggered a volley of action potentials in the cervical vagus with a mean delay of 27 ± 2 ms (n = 4) (Figures 1D and 1E). Calculated conduction velocity of 0.8 m/s was consistent with the properties of the rat DVMN cardiac vagal pre-ganglionic neurons that have C-fiber axons (36). Stimulation of DVMN neurons expressing ChIEF with 10-ms pulses applied at a frequency of 15 Hz led to a robust increase in vagal efferent activity and was associated with a reduction in heart rate (by 30 ± 12 beats/min; 8.5% reduction; p = 0.037) and mean ABP (by 15 ± 4 mm Hg) during the period of stimulation (Figure 1D).

The average infarct size in the post-MI/shamVNS group was 33.4 ± 1.6 (n = 13); in the post-MI/optoVNS group it was 29.9 ± 1.3 (n = 19; p = 0.09). Four animals in the post-MI/shamVNS group and 7 animals in the post-MI/optoVNS experimental group developed small infarcts (<30%). In accord with the study design, the data obtained in 9 animals in the post-MI/shamVNS group and 12 animals in the post-MI/optoVNS group that developed infarcts ≥30% were included in the analysis. There was no difference in mean infarct size between the 2 groups (p = 0.14) (Figure 2A).

Exercise capacity was significantly lower in post-MI rats expressing eGFP in the DVMN compared with sham-operated animals (0.28 ± 0.03 kJ in post-MI/shamVNS group vs. 0.56 ± 0.08 kJ in sham/shamVNS group; p = 0.039) (Figure 2B). Optogenetic stimulation of the DVMN neurons expressing ChIEFtdTomato enhanced the exercise capacity in sham-operated animals (1.05 ± 0.12 kJ in sham-operated rats expressing ChIEFtdTomato vs. 0.56 ± 0.08 kJ in sham-operated rats expressing eGFP; p < 0.001) (Figure 2B) and improved the exercise capacity in animals with LAD occlusion.
TABLE 1 Hemodynamic and Other Group Data

|                          | Sham MI | Post-MI |
|-------------------------|---------|---------|
|                         | shamVNS (n = 8) | optoVNS (n = 9) | shamVNS (n = 9) | optoVNS (n = 12) |
| Body weight (g)         | 431 ± 18 | 406 ± 29 | 453 ± 18 | 391 ± 5 |
| Infarct size            | –       | –       | –       | –       |
| HR (beats/min, under isoflurane) | 348 ± 9 | 380 ± 12 | 364 ± 12 | 389 ± 12 |
| Ejection fraction (%)   | 49.6 ± 5.1 | 76.3 ± 2.3 | 32.7 ± 3.0 | 49.3 ± 3.3 |
| E/A ratio               | 1.2 ± 0.1 | 1.6 ± 0.1 | 0.9 ± 0.1 | 1.2 ± 0.1 |
| dP/dt\text{min} (mm Hg/s) | –6,136 ± 269 | –6,774 ± 391 | –4,348 ± 275 | –5,550 ± 176 |
| Pulse pressure (mm Hg)  | 52 ± 4  | 51 ± 2  | 36 ± 4  | 51 ± 5  |
| LV to body weight ratio | 2.12 ± 0.07 | 1.92 ± 0.06 | 2.19 ± 0.08 | 2.24 ± 0.05 |
| RV to body weight ratio | 0.38 ± 0.01 | 0.37 ± 0.01 | 0.42 ± 0.02 | 0.36 ± 0.02 |
| Lung fluid (%)          | 77.5 ± 0.3 | 77.8 ± 0.5 | 77.3 ± 0.3 | 78.4 ± 0.3 |

Values are mean ± SEM. dP/dt\text{max} = maximum rate of left ventricular pressure decrease; HR = heart rate; LV = left ventricle; MI = myocardial infarction; RV = right ventricle; VNS = vagus nerve stimulation.

The data obtained in the present study showed that optogenetic stimulation of vagal pre-ganglionic neurons of the DVMN (optoVNS) for 15 min every 48 h over 4 weeks following LAD occlusion preserved LV systolic function and maintained exercise capacity in a rat model of heart failure. These data are consistent with the results of earlier studies that showed that optogenetic stimulation of the DVMN neuronal activity protects the heart against acute ischemia/reperfusion injury in a rat model of MI (11), and also increases LV contractile responses to \( \beta \)-adrenoceptor stimulation and improves exercise capacity in healthy rats (24). Together, these data suggested that stimulation of vagal efferent activity originating from the DVMN might be critically important to confer the therapeutic benefit of VNS in heart failure.

Cardiac vagal efferent activity originates from populations of vagal pre-ganglionic neurons residing in 2 brainstem nuclei: the DVMN and the nucleus ambiguus (37,38). Control of pacemaker tissue, and thus, heart rate is provided predominantly by neurons of the nucleus ambiguus. Although most of the DVMN neurons innervate the visceral organs, a subpopulation of these cells with cardiac projections provides functional innervation of the LV and modulates its electrical (39) and contractile properties (27). Activity of DVMN neurons has a relatively small effect on heart rate (37).

The intermittent stimulation of the DVMN (which lacks major chronotropic effects) preserved cardiac function in rats with MI (as shown in this study), which was consistent with the results of some previous reports that suggested that the beneficial effects of VNS in heart failure were not entirely dependent on lowering heart rate (13-15). This was an important...
conclusion to draw in the context of a strong clinical association between low chronotropic vagal tone and the risk of death after MI or in established heart failure (40,41), and of the effectiveness of pharmacological interventions that lower heart rate (e.g., β-blockers and I₈ inhibitors) (42–44). In most clinical trials of VNS in heart failure, the stimulating electrodes were placed on the right cervical vagus nerve, with the aim of lowering the heart rate, but it was not reliably attained (20).

Several mechanisms that might underlie the beneficial effects of VNS on the failing heart were proposed, including improvements of autonomic balance via sympathetic inhibition, inhibition of renin-angiotensin system activity, and anti-inflammatory effects of vagus stimulation (5,45). Selective efferent VNS applied in this study was likely to activate some or all of these mechanisms, whereas the observed effects of optogenetic stimulation of the DVMN on myocardial function in sham-operated animals suggested a plausible additional and/or alternative mechanism. Surprisingly, the effect of long-term VNS on a healthy heart has never been described, because the design of all of the preceding studies that investigated the effect of VNS in heart failure (12–15,17) excluded the experimental group(s) of healthy subjects (“sham heart failure” groups) receiving the stimulation. In this study, we observed proportionally similar improvements in key measures of LV systolic function and exercise capacity in groups of rats with permanent LAD occlusion and in sham-operated animals. These data were consistent with the previously reported results that showed that optogenetic stimulation of DVMN neuronal projections enhanced myocardial contractility and responsiveness of the ventricular myocardium to β-adrenoceptor stimulation (24). These effects of DVMN stimulation were associated with reduced

![Summary data illustrating the values of (A) the maximum first differential of LV pressure (LV dP/dtmax), (B) LV end-systolic pressure (LVESP), (C) mean arterial blood pressure (MAP), and (D) LV end-diastolic pressure (LVEDP) in rats transduced to express eGFP or ChIEFtdTomato by the DVMN neurons after 4 weeks of light stimulation commencing 2 days after the occlusion of the left anterior descending artery or sham surgery. Data are presented as individual values and mean ± SEM. Comparisons are made using 2-way analysis of variance followed by Sidak’s correction for multiple comparisons. Abbreviations as in Figures 1 and 2.]
myocardial expression of G-protein–coupled receptor kinase 2 (GRK2) and β-arrestin 2 (24). GRKs phosphorylate β-adrenergceptors and recruit arrestins to block receptor coupling to G-proteins, which leads to receptor desensitization and internalization (46). Increased expression and activity of GRKs contribute to the progressive decline of myocardial contractile function in heart failure (47). Therefore, inhibition of GRKs was proposed as a potential therapeutic strategy of heart failure treatment (47). We recently proposed (28) that GRK2 and arrestin expression in ventricular cardiomyocytes is under parasympathetic control, with vagal withdrawal (e.g., in heart failure) leading to upregulation and enhanced vagal activity (e.g., in exercise training) leading to down-regulation of expression, with opposite changes in contractility.

Cardiac projections of a subset of the DVMN neurons innervate the LV and modulate its electrical (29) and contractile properties (27). It is plausible that optogenetic stimulation of the DVMN activates the neuronal pathways and ventricular molecular mechanisms similar to that recruited by cardiac contractility modulation, which is a novel therapeutic approach of heart failure treatment that involves application of an electrical current during the ventricular absolute refractory period (48,49). The high voltage (~7.5V) applied in cardiac contractility modulation would be sufficient to capture the autonomic nerves, including parasympathetic fibers innervating the ventricular myocardium (50).

If this hypothesis is correct, then the VNS-based heart failure therapy should aim at stimulating the activity of vagal efferent fibers innervating the ventricles. VNS devices used in clinical trials conducted to date were not developed with an attempt to recruit specific vagal projections. Electrical stimulation applied to the cervical vagus preferentially activates sensory fibers because they have a lower activation threshold than efferent fibers (51). Experiments in large animals (pigs and dogs) demonstrated that low current stimulation (up to ~2 mA) recruits afferent fibers predominantly and activates autonomic reflex pathways, which lead to increases in heart rate due to inhibition of vagal activity centrally (52,53). Application of higher VNS currents (~2.5 mA) is required for stimulation of efferent fibers to achieve vagally mediated lowering of the heart rate (53). No changes in heart rate in response to electrical VNS are observed when the effects of afferent and efferent fiber stimulation are in balance. These studies led to the development of a concept of a “neural fulcrum,” which was defined as the operating point based on the frequency-amplitude-pulse width of VNS, in which the stimulation has no effect on heart rate (51).

The efficacy of VNS applied within the neural fulcrum is currently being tested in the ongoing ANTHEM-HFREF trial (54). Because recruitment of efferent fibers requires more aggressive stimulation, simultaneous capture of vagal afferents may lead to significant side effects, including dysphonia, neck pain, and cough, as reported by the investigators of the NECTAR-HF trial, in which no beneficial effect of VNS on LV function was observed (20).

CONCLUSIONS

The data obtained in this study suggest that stimulation of vagal efferent innervation of the ventricles might be critically important to deliver the therapeutic benefit of VNS in chronic heart failure.

A revised approach to the electrical VNS may be necessary to minimize the side effects that currently restrain the intensity of stimulation required for the effective recruitment of cardiac vagal efferent activity. The data reported here might be important for further development of the electrical VNS technology for targeted stimulation of a select group or subset of fibers within the trunk of the vagus nerve. This would also require detailed functional anatomical mapping of the fascicular organization of the human cervical vagus (22); this work is ongoing. Although optogenetic techniques are currently limited to preclinical research, advances in human gene therapy point the way toward eventual clinical applications of the technology. VNS using light (optoVNS) can be applied at the cervical level, and thus, can circumnavigate limitations posed by the complex anatomy of the vagus nerve and nonspecific nature of the whole nerve electrical stimulation.

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COMPELENCY IN MEDICAL KNOWLEDGE: VNS has been shown to slow the progression of myocardial remodeling and dysfunction in animal models of chronic heart failure. However, several multicenter clinical trials designed to test the efficacy of VNS in large cohorts of patients with heart failure did not demonstrate similar benefits with respect to the primary clinical endpoints. The nonselective nature of VNS, delivered by implantable stimulators placed on the nerve at the cervical level, may account for the failure to translate promising results of preclinical studies. To maximize the efficacy and limit the side effects, further development of VNS as a potential treatment for heart failure may require methods that allow selective stimulation of specific groups or subsets of vagal fibers.

TRANSLATIONAL OUTLOOK: Stimulation of vagal efferent projections to the ventricle may be critically important to deliver the therapeutic benefit of VNS in heart failure. A refined approach to VNS may be necessary to minimize the side effects that currently restrain the intensity of stimulation, which is required for the recruitment of cardiac vagal efferent innervation. Although optogenetic techniques are currently limited to research in animals, advances in human gene therapy point the way toward eventual clinical applications of the technology. VNS using light (optoVNS) can be delivered at the cervical level, and therefore, can circumnavigate limitations posed by the complex anatomy of the cervical vagus nerve and the nonspecific nature of the whole nerve electrical stimulation.

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Cardiac Sympathetic-Parasympathetic Interaction
The Endless Story of Yin and Yang*

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In Ancient Chinese philosophy, yin and yang express a concept of dualism, underlying how seemingly opposite or contrary forces may actually be complementary, interconnected, and interdependent in the natural world. This concept perfectly applies to cardiovascular autonomic control, at least in physiological conditions. Sympathetic and parasympathetic efferent neurons tightly collaborate in a closed-loop system to constantly adjust cardiac output to body demands based on sensory feedback and feedforward control loops and complex integrative mechanisms. In pathological conditions, when the homeostatic capability of the system is overcome, maladaptive responses occur, leading, in the great majority of cases, to excessive sympathoexcitation coupled with reduced parasympathetic output. As opposed to the classical theory of the centrally determined cardiac neuronal command, it is now well established that the first integration of cardiac sensory inputs occurs at the peripheral level, within interneurons of the intrinsic cardiac ganglia; these ganglia constitute the so-called intrinsic cardiac nervous system, whose structural and functional integrity is crucial to preserve cardiac function. Parasympathetic postganglionic neurons within each ganglion receive bilateral projection via the vagal nerves and in turn project to broad areas of preferential, albeit not exclusive, influence within the heart. Of note, despite the antiarrhythmic properties of vagal nerve stimulation (VNS) were first anecdotally reported in 1859, it was more than 100 years later that the existence of parasympathetic projections to the ventricles was first demonstrated. Left VNS acutely decreased left ventricular (LV) contractility and relaxation rate in vivo both in animal and in human paced hearts. The acute negative inotropic effect was blocked by muscarinic antagonism and attenuated by β-blockade, and the same was found for the increase in ventricular effective refractory period produced by VNS. The reduction in ventricular inotropism and the increase in effective refractory period duration in normal hearts were quantitatively related, underlying the importance of autonomic influences to regulate electromechanical coupling; ICaL inhibition by cholinergic muscarinic receptor signaling is thought to play a pivotal role in these effects. Nonetheless, the existence of a tonic inhibitory muscarinic influence on cardiac inotropy has long been questioned.

In 2016, Machhada et al. (1) demonstrated that atropine systemic administration under β-blockade and C1 level spinal cord transection increases LV contractility of anaesthetized rats. Preganglionic parasympathetic neurons of the nucleus ambiguous and the vagal dorsal motor nucleus (DVMN) were suggested to have a preferential control over pacemaker and ventricular tissue, respectively. From an anatomical point of view, canine studies showed that most efferent vagal fibers to the LV first cross the
atroventricular groove and then dive intramurally to
the subendocardium, as opposed to efferent sympa-
thetic fibers located throughout their course in the
subepicardium along with coronary arteries. Both
sympathetic and parasympathetic fibers run from the
base to the apex of the heart; their different
anatomical course originally led to speculate that
parasympathetic dysfunction after myocardial
infarction (MI) might be the result of subendocardial
injury. This hypothesis is in overt disagreement with
several preclinical studies showing beneficial effects
of electrical right VNS applied after healed MI on
overall survival, LV function, and ventricular ar-
rhythmas susceptibility (2). Despite the large amount
of data on the sympathetic component, information
about chronic parasympathetic remodeling after MI
has long been lacking. In 1988, Inoue and Zipes (3)
demonstrated that a functional parasympathetic
denervation occurs, in addition to the sympathetic
one, distally to a transmural MI and within 180 min of
coronary legation. Almost 30 years later, using a
chronic post-MI porcine model, Vaseghi et al. (4)
showed that in contrast to norepinephrine levels,
cardiac acetylcholine levels remain preserved 6 to
8 weeks after MI in border zones and in viable
myocardium of infarcted hearts. Yet, in vivo neuronal
recordings from postganglionic parasympathetic
neurons demonstrated abnormalities in resting firing
frequency and in responses to stimuli. Overall, these
data prove that parasympathetic cardiac neuronal
network is anatomically intact but profoundly
dysfunctional after a healed MI, therefore reinforcing
the strong pathophysiological rationale for therapeu-
tic interventions aimed to restore a proper cardiac
vagal output. Of note, in the setting of electrical VNS
a preferentially efferent, rather than afferent, fiber
activation has been shown to depend on the shape
and the orientation of the stimulating electrode as
well as the frequencies, pulse widths, and currents
used (5). Finding the most effective stimulation pa-
rameters is both challenging technically and
extremely cumbersome to investigate clinically.
Moreover, because an abnormal cardiovascular
afferent signaling significantly contributes to the
condition of autonomic imbalance, questions have
been raised about the potential contribution of
afferent mechanisms to the beneficial effects of
electrical VNS observed in preclinical studies.

In this issue of JACC: Basic to Translational Science,
Machhada et al. (6) have used the elegant technology
of optogenetic stimulation to assess the effects of a
4-week program of intermittent (15 min every 48 h)
DVMN neurons activation on LV contractility and
exercise capacity in normal and in post-MI rats. Four
groups were studied in order to guarantee that both
experimental conditions (sham-MI and post-MI rats)
had a control arm, represented by animals implanted
with the optogenetic electrode but insensitive to blue
light-induced DVMN activation. Echocardiography,
direct hemodynamic assessment, and exercise stress
test were performed in all groups at the end of the
study period, therefore precluding the possibility to
assess optogenetic VNS induced changes within the
same group. In post-MI rats, despite an infarct size of
at least 30% of LV mass, optogenetic VNS improved LV
systolic function and exercise capacity as compared
with post-MI rats with a similar infarct size not
receiving VNS. In sham-MI animals, the group who
received optogenetic VNS showed a better LV systolic
function and exercise capacity, with no differences in
resting mean arterial pressure. The favorable results
obtained in post-MI rats agree with previous preclini-
cal studies of electrical VNS in both ischemic and
nonischemic heart failure models pursuing a prefer-
entially efferent stimulation. Antiadrenergic (at both
pre- and post-synaptic levels) as well as muscarinic
and nicotinic cholinergic mechanisms of action for
efferent VNS have been consistently demonstrated in
models of cardiac pathology. Of note, as compared
with the landmark study of Li et al. (7) (with electric
stimulation of the right cervical vagus nerve), in this
study optogenetic VNS was applied earlier after MI
e.g., 48 h vs. 14 days after coronary artery ligation) at
a time when acute cardiac remodeling is expected to
be still ongoing. The observed results, namely an
almost normal LV systolic function 4 weeks after a
large MI, are significant and reinforce the patho-
physiological concept that the sooner the cardiac
vagal output is restored, the better the outcome may
be. The potential for an additional benefit of afferent
mechanisms cannot be answered by this study but can
be hypothesized based on the reduction of ischemia-
reperfusion damage and related arrhythmias demon-
strated by transcutaneous auricular VNS.

The improvement in LV function caused by opto-
geretic VNS in sham MI is rather unexpected and
worth discussing. In normal animals that have no
reason to be characterized by autonomic imbalance,
chronic optogenetic VNS of DVMN may potentially
produce effects similar to those of physical training,
with the important difference of a preferential effect
on ventricular control as opposed to the combined
effect of training on pacemaker and ventricular tis-
sue. Because cardiac vagal activity is not completely
abolished during physical effort at submaximal
levels of exercise, an increased vagal output during a
high-level background adrenergic activity might
protect cardiomyocytes from cytoplasmatic calcium
overload and improve calcium transient for contraction. Yet, this mechanism cannot explain the increased LV systolic function observed at rest. It must be acknowledged that same group had previously demonstrated that optogenetic stimulation of DVMN neurons in normal rats enhances myocardial contractility and responsiveness to β-adrenoceptor stimulation. These effects were associated with reduced myocardial expression of GRK2 (G protein-coupled receptor kinase 2) and β-arrestin 2, which are both involved in β-adrenergic receptor desensitization and internalization. Notably, β-arrestin 2 has also been identified as one of the key regulatory molecules involved in length-dependent enhancement of cardiac myofilament Ca2+ sensitivity (Frank-Starling mechanism) (8). Overall, parasympathetic overactivity mediated decrease in β-arrestin 2 may thus potentially lead to increased sympathetic responsiveness and less reliance on the Frank-Starling mechanism. Because parasympathetic DVMN neurons have been shown to preferentially control the ventricles, rather than the pacemaker area, this specific approach minimizes the heart rate lowering that would lead to increased cardiac filling and recruitment of the Frank-Starling mechanism (Figure 1). Intriguingly, back in 1986 (9), one of us demonstrated that older as compared with younger subjects had a higher percent increase in cardiac output during graded submaximal effort, despite a similar heart rate and mean arterial pressure response. A greater contribution of the Frank-Starling mechanism during exercise in older subjects was proposed to explain these findings. This hypothesis is in good agreement with the current previously mentioned data, considering that a preferential reduction of vagal output at the ventricular level is also suggested by the recently reported deterioration of parasympathetic DVMN neurons that occurs with aging. These findings are certainly worth further investigation.

**Figure 1** Simplified Working Interpretation of β2-arrestin Activation Impact on Ventricular Inotropism Based on Published Data

β2-arrestin is coupled to both G protein-coupled receptors (such as β-adrenoceptor) and not G protein-coupled receptors such as the epidermal growth factor receptor (EGFR). As detailed in the paper, Machhada et al. (6) recently proposed that β2-arrestin and G-protein-coupled receptor kinase 2 (GRK2) expression in ventricular cardiomyocytes is under parasympathetic control, with vagal withdrawal leading to upregulation, and enhanced vagal activity downregulation of expression. Once activated, GRK2 phosphorylate β-adrenoceptors and recruit β2-arrestin to block receptor coupling to G-proteins, leading to receptor desensitization and internalization. Additionally, other groups demonstrated that angiotensin II type 1 receptors (AT1Rs) can function as mechanosensors with a ligand-independent mechanism to activate β2-arrestin-dependent signaling and that both β2-arrestin and AT1Rs are key regulatory molecules in the Frank-Starling mechanism (8).

AC = adenylyl cyclase; β1-AR = β-adrenoceptor; ERK = extracellular regulated kinase; GRK = G-protein coupled receptor kinase; MAPK = mitogen-activated protein kinase; P = phosphorylation site; PKA = cAMP-dependent protein kinase A.
Overall, the present study by Machhada et al. (6) reinforces the rationale for efferent VNS at the ventricular level in heart failure. It also draws attention to a novel additional pathway that might be involved in sympathetic-parasympathetic interaction at the cardiac level.

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Development of Tissue Engineered Heart Valves for Percutaneous Transcatheter Delivery in a Fetal Ovine Model

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HIGHLIGHTS

- A fully biodegradable fetal valve was developed using a zinc-aluminum alloy stent and electrospun PCL leaflets.
- In vitro evaluation of the valve was performed with accelerated degradation, mechanical, and flow loop testing, and the valve showed trivial stenosis and trivial regurgitation.
- A large animal model was used for percutaneous delivery of the valve to the fetal pulmonary annulus.
- Following implantation, the valve had no stenosis or regurgitation by echocardiography, and the fetal sheep matured and was delivered at term with the tissue-engineered valve.

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Congenital cardiac anomalies represent the most common birth defect, affecting approximately 0.9% of all live births (1). Despite significant advances in surgical and medical management, they remain a leading cause of death in the newborn period and lead to lifelong morbidity in survivors (1). Most major congenital cardiac anomalies require reconstructive surgery, often requiring the use of man-made, xenographic, or homograft material in the form bioprosthetic heart valves and valved conduits. Use of these valves and conduits is a significant source of morbidity and reoperations, owing to biocompatibility issues (2). For example, bioprosthetic valves and conduits are subject to conduit contracture and shrinkage, tissue degeneration, calcification, immunologic response consistent with cellular rejection, and somatic outgrowth, leading to the need for multiple valve replacements (3). This is particularly true for valve replacements in young children. When valved conduits are implanted in infants and young children, the median time to the next conduit replacement is only 7.5 years, and right ventricular (RV) outflow tract reconstructions in patients under 2 years of age have a 5-year freedom from intervention of 46.1%, demonstrating a need for improved valves for these patients (4,5).

Tissue engineering provides a potential strategy for creating better biomaterials for use in reconstructive cardiac operations. Using tissue engineering techniques, replacement heart valves can be made from a biodegradable scaffold and an in-growth of the recipient’s own cells. Over time, the resulting autologous neovalves become living structures with the ability to grow, repair, and remodel. The regenerative capacity of the fetal milieu makes it a prime target for tissue regeneration and neovalve formation. This unique environment was the impetus for the development of fetal cardiac interventions. Fetal pulmonary balloon valvuloplasty is performed in fetuses with pulmonary atresia and intact ventricular septum or near-pulmonary atresia and intact ventricular septum in an attempt to allow the right heart to remodel enough to support a biventricular circulation after birth (6). However, of 21 fetuses who underwent technically successful or partially successful fetal interventions, almost all had restenosis during later gestation, and 4 had reatresia (6).

Fetal pulmonary valve replacement has the potential to prevent restenosis and reatresia from occurring. Weber et al. (7) implanted 9 tissue-engineered heart valves (TEHVs) sewn into self-expanding nitinol stents and delivered into the ovine fetal pulmonary annulus through a fetal thoracotomy and purse-string suture in the RV using a 14-F (4.7 mm) delivery system. Nitinol does not have growth capacity and needs a large-profile delivery system, requiring uterine and fetal incisions. A fully bioabsorbable
stent for valve delivery is needed to allow growth and remodeling with the patient throughout fetal life and childhood.

We sought to construct the first completely bioabsorbable TEHV for fetal percutaneous and transcatheter implantation in the pulmonary valve position. We modeled the TEHV after currently available transcatheter techniques for replacing pulmonary and aortic valves in children and adults (8,9). In this manner, balloon-expandable valves are sewn into a stent, crimped onto an angioplasty balloon, and delivered through a catheter into the existing degenerated valve. Inflation of the angioplasty balloon expands the stent within the valve annulus, thereby pinning the existing degenerated leaflets between the stent and the annulus. Despite its technical challenges, the development of a percutaneous, transcatheter delivery system for TEHV replacement in utero would be superior to utilization of open fetal surgical methods, owing to the lower incidence of inducing labor to utilization of open fetal surgical methods, as well as obviating the need for uterine and fetal incisions (10). In this work, we present a multidisciplinary approach to develop a completely bioabsorbable TEHV that is delivered through a percutaneous and transcatheter approach to the fetal pulmonary annulus in a large animal model.

METHODS

STENT DEVELOPMENT. In order to create the completely bioabsorbable structure, a bioabsorbable metal alloy stent was used, which was previously shown in a rabbit model to be an excellent bioabsorbable stent material candidate (11). A zinc-aluminum alloy of 96% zinc and 4% aluminum (Zn-4Al) was used to manufacture the stents. Zn-4Al ingots were cast at Michigan Tech University (Houghton, Michigan) and placed under a repetitive thermal processing to achieve the desirable fine alloy microstructure (Fort Wayne Medical, Fort Wayne, Indiana). The ingots were then extruded into ~12-mm rods, which were machined to 10 mm in length. The rods were then drawn into 1.8-mm outer diameter tubes with an average wall thickness of 0.151 mm. The tubes were laser cut into a proprietary closed-cell stent design to minimize stress and strain during balloon inflation. The stents were electropolished and sterilized with low-temperature ethylene oxide gas. The estimated foreshortening at an inflation diameter of 7 mm was ~20%, with resulting final length of ~8 mm following deployment.

ELECTROSPINNING. The valves were constructed of electrospun polycaprolactone (PCL) (molecular size \([M_n] = 80,000\) Da (Sigma-Aldrich, St. Louis, Missouri)). Electrospinning solution was prepared by dissolving PCL in an aqueous 2,2,2-trifluoroethanol (Sigma-Aldrich) mixture (volume ratio of 2,2,2-trifluoroethanol to deionized water = 5:1) at 14 wt/vol%. PCL was electrospun onto a rotating mandrel (6.35-mm diameter) wrapped with aluminum foil (Boardwalk Essendant, Deerfield, Illinois), using the following conditions: applied voltage = ±12 kV, needle size = 22 gauge, distance between needle and aluminum collector = 30 cm, distance between the mandrel and aluminum collector = 10 cm, PCL solution infusion velocity = 29 µl/min, mandrel angular speed = 80 rpm. The thickness of the PCL tubular scaffold was controlled in a targeted range of 50 to 100 µm by measuring the mandrel under high-speed laser micrometer (LS-7070M; Keyence Corp., Itasca, Illinois) (12).

TEHV CONSTRUCTION IN THE STENT. To construct the TEHV in the zinc stent, the electrospun PCL tube was fashioned into a valved-conduit using a tube-within-a-tube technique (13). The stent was first expanded using a commercially available low profile 6 mm balloon catheter. A 6 mm × 8 mm PCL tube was then placed in the stent lumen and positioned within the proximal two-thirds of the stent. Using a 10-0 mono filament suture, the PCL tube was attached to the proximal end of the stent with interrupted stitches circumferentially. The PCL was then sutured in 3 locations inside of the zinc stent to form a biodegradable trileaflet TEHV (Figure 1).

VALVE STRUCTURAL CHARACTERIZATION. ImageJ 2.0.0 (National Institutes of Health, Bethesda, Maryland) and an image analysis-based structural characterization algorithm previously developed (14) were used to characterize the PCL fiber network. Scanning electron microscopy (SEM) images of 500× (n = 7) and 1000× (n = 7) were analyzed and fibrous mesh characteristics including fiber tortuosity, alignment and density were quantified. Tortuosity was measured using ImageJ through tracing the total and end-to-end fiber lengths of a representative 12 fibers in each SEM image, with tortuosity calculated as:

\[
\text{Tortuosity (T)} = \frac{\text{Total Fiber Length}}{\text{End to End Fiber Length}}
\]

For fiber alignment, the normalized orientation index (NOI) was chosen as a representative measurement and was calculated using the previously
mentioned structural characterization algorithm. An NOI is defined as:

\[ NOI = \frac{90 - OI}{90} \times 100\% , \quad NOI \in [0\% , 100\%] \]

where the OI (orientation index) is defined as the angle range containing the orientation of 50% of all fibers (14). The algorithm calculates NOI by creating a skeletonization of the fibers in the mesh, measuring the orientation angle of each fiber detected, and calculating an orientation distribution function. The OI is then calculated as the angle range containing half of all fibers centered on the main angle of orientation. After normalization, the NOI represents a single value corresponding to fiber alignment, with an NOI value of 0% representing no fiber alignment and 100% representing very high fiber alignment.

Linear fiber density was calculated from SEM images at 1,000× magnification using the structural characterization code as:

\[ \xi = \frac{\text{Total Fiber Length}}{\text{Total Area}} \]

**ACCELERATED DEGRADATION.** Dried samples of the electrospun PCL underwent accelerated degradation testing by using a high-pH solution to accelerate the natural hydrolysis degradation mechanism of the PCL polymer. The samples were massed, rinsed in 100% ethanol, and submerged in 1-mL vials of 6-M sodium hydroxide at room temperature. At the end of the degradation time, samples were rinsed twice with dH2O, frozen to -80°C, and lyophilized overnight. Dry masses at the end of degradation were compared with pre-degradation masses to determine remaining mass, and samples were then processed for scanning electron microscopy or gel permeation chromatography.

**GEL PERMEATION CHROMATOGRAPHY.** The changes of molecular weight distributions of the PCL scaffold through the accelerated degradation duration were characterized by gel permeation chromatography (OMNISEC GPC/SEC system; Malvern Panalytical, Malvern, United Kingdom), equipped with a light scattering detector and differential refractive index detector. A single-pore column with 70,000-Da exclusion limit and a general-purpose mixed-bed column with 20,000,000-Da exclusion limits (Viscotek T-3000 and D-6000M, 300 × 8.0 mm dimensions; Malvern Panalytical) were used for separation, along with tetrahydrofuran used as stationary and mobile phases, respectively. A total of 1 mg/ml of each
polymer sample in tetrahydrofuran solution was prepared and filtered through a 0.2-μm syringe filter before testing. Polystyrene was used as a standard for molecular weight calibration with a concentration of 1 mg/ml. Weight average molecular weight (Mw), number average Mn, and polydispersity index (PDI), a measure of the heterogeneity within the sample defined as Mw/Mn, were evaluated using OMNISEC software.

**SCANNING ELECTRON MICROSCOPY.** Samples were mounted on SEM mounts using double-sided carbon tape, and sputter coated with gold under Argon gas to 3 nm. Samples were then imaged on a Hitachi S4800 SEM (Hitachi, Tokyo, Japan) at 5 kV.

**BIAXIAL MECHANICAL TESTING.** To perform the biaxial testing, 5 × 5 mm specimens were excised and mounted in an orientation perpendicular to the axial direction of the PCL tube. A 2 × 2 array of small, black markers, measuring approximately 50 to 150 μm in diameter, were used to mark each specimen in the central 1 × 1 mm region of the specimen. The markers were applied on the specimens using a STAEDTLER pigment lining pen (STAEDTLER, Nuremberg, Germany). All testing occurred in deionized water at room temperature. Each specimen underwent 9 test protocols. For each protocol, a prescribed deformation was applied to the specimen using 12 independent actuators. The markers were used to measure the deformation gradient and provide feedback to the control system. Six load cells were used to capture force measurements as the specimen was deformed. The resulting first Piola-Kirchhoff stress tensor was calculated for each protocol. The material’s response to deformations was determined using 9 protocols. First, the material’s response to axial deformations was characterized. In the first protocol, a constant equibiaxial ratio of deformation was applied (F11:F22) = 1.1:1.1 for 10 cycles. The next 4 protocols applied 5 cycles of axial deformation of varying ratios of F11 to F22 equal to 1.033:1.1, 1.02:1.1, 1.1:1.033, and 1.1:1.02, respectively. Next, the material’s response to shear deformations was characterized. Three protocols applied 5 cycles of different types of shear deformation equal to -0.15 in the F12, F21, and F12 and F21 directions, respectively. This allowed for a broad characterization of the shear response of the specimens. Finally, the last protocol applied both maximum biaxial deformation and shear deformation: F11 = F22 = 1.1 and F12 = F21 = -0.15. Images were taken of the specimen both before and after testing to observe any inelastic effect on the material due to the mechanical testing.

**HEMODYNAMIC ASSESSMENT.** A hemodynamic evaluation of the prototype TEHV was performed using a dedicated right heart pulse duplicator, similar to previous studies (15–17). Briefly, the pulse duplicator was composed of a reservoir to mimic atrial function, a bladder pump controlled by compressed air to simulate ventricular function, and a mechanical valve between the reservoir and the pump that functioned as an atrioventricular valve. The duplicator also included a pulmonary valve chamber with an annulus of 8 mm, where the valve was deployed, a compliance chamber that simulated pulmonary vascular compliance, and a flow valve to set the pulmonary capillary resistance in order to control the cardiac output or mean flow rate through the fetal heart valve. A working fluid of 60/40 water to glycerin was used as a blood analog to provide a density of 1,060 kg/m³ and kinematic viscosity of 3.5 × 10⁻⁶ m²/s. A flow probe (HXL; Transonic Inc., Ithaca, New York) allowed the reading of the average flow rate in the system as well as the flow waveform. The cardiac output was set at 0.5 l/min with a pulmonary artery pressure of 28/4 mm Hg, and the heart rate was set at 90 beats/min (18–20). One hundred consecutive cycles of flow rate and transvalvular pressure gradient (PG), measured using pressure transducers (Validyne Engineering Corp., Northridge, California), were acquired at a sampling frequency of 100 Hz, in accordance with previous studies (16,21,22). From these data, common measures of in vitro valve performance, including effective orifice area (EOA), regurgitant fraction (RF), and pinwheeling index, were computed.

EOA, a measurement of the effective jet area during the phase of the cardiac cycle in which the valve is fully open (23), was calculated based on Gorlin’s equation as follows:

$$EOA = \frac{Q}{5.16 \sqrt{PG}}$$  \hspace{1cm} (1)

where Q represents the root mean square pulmonary valve flow (cm³/s) and PG (mm Hg) is the mean transvalvular pressure gradient over a complete cardiac cycle.

The RF was calculated as the ratio of the closing volume (CV) and leakage volume (LV) to the forward flow volume (FV), in accordance with ISO 5840-3 (24):

$$RF = \frac{LV + CV}{FV}$$ \hspace{1cm} (2)

Pinwheeling, or localized bending of leaflet material upon closure, is known to cause increased localized bending stresses and hypothesized to correlate with decreased durability (25,26). Ideally, no
pinwheeling or a ratio of 0 is for lealet and valve durability. The pinwheeling index was computed from en face still frames obtained from high-speed imaging, as per the following equation and in accordance with previous publications (15):

$$PI = \frac{L_{\text{actual}} - L_{\text{ideal}}}{L_{\text{ideal}}}$$  \hspace{1cm} (3)

where $L_{\text{actual}}$ represents the deflected free edge of the lealet in the closed position and $L_{\text{ideal}}$ represents the unconstrained ideal configuration (or shortest possible distance from post and central coaptation region) of the lealet free edge.

**ANIMAL CARE AND USE.** All procedures were evaluated and approved by the Institutional Animal Care and Use Committee, following humane guidelines as outlined by the National Institutes of Health. Although there are no current large animal models of left or right heart congenital heart disease, pregnant sheep have been shown to be an excellent surrogate for fetal surgical research, owing to similar cardiovascular anatomy and fetal size, compared with humans (27). Pregnant Cheviot ewes between 109 and 115 days gestation (term 145 to 151 days) (28) were used for the study, with 1 to 3 fetuses each. After overnight fasting, the ewes were sedated with
intravenous 5-mg/kg bolus of propofol, placed supine, and intubated. They were ventilated with 100% oxygen and 1% to 2% isoflurane during the procedure.

**Fetal Ovine Percutaneous Transcatheter Pulmonary Valve Replacement.** A custom-made 7 mm × 12 mm TYSHAK Mini Pediatric Valvuloplasty Catheter (NuMED, Hopkinton, New York) was placed over a 0.014 inch × 190 cm Hi-Torque All Star guidewire (Abbott, Abbott Park, Illinois). A Touhy-Borst sidearm adapter was placed on the hub of the balloon catheter. The 0.014-inch wire was marked with 5 cm protruding from the end of the catheter, by securing a torque device behind the Touhy-Borst adapter. The catheter and wire were placed through the outer blunt-tipped cannula of a 17-gauge (outer diameter 1.5 mm) and 15-cm Universal Coaxial Introducer Needle (BD, Franklin Lakes, New York) with the trocar removed, and the catheter was marked with a sterile pen at the point at which the wire was at the tip of the 17-gauge cannula, the point at which the balloon tip was at the tip of the cannula, and the point at which the entire balloon was protruding from the cannula tip. A 10-mm-long zinc alloy stent, with or without the TEHV sewn into it, was crimped on the center of the balloon, without a negative preparation, using a manual crimper on the tightest setting, until the balloon catheter with either the stent or TEHV fit through the 17-gauge cannula without resistance (Figure 2). The 3 facets of the 17-gauge trocar were scraped with a #11 scalpel blade to increase echogenicity.

A combination of abdominal fluoroscopy and ultrasound was used to determine the number and locations of the fetuses. Under ultrasound imaging, a 20 gauge × 20 cm Chiba biopsy needle (Cook Medical, Bloomington, Indiana) was inserted into the fetal leg, and an intramuscular injection of fentanyl 50 µg/kg, atropine 20 µg/kg, and vecuronium 200 µg/kg was administered (fetuses were assumed to weigh 1,500 g, based on weights obtained at necropsy). A 22 gauge × 20 cm Chiba biopsy needle was used to test the trajectory needed to enter the fetal right ventricle from the ewe’s abdominal wall. The fetal chest was not entered with this needle. In some cases, a small Pfannenstiel (low transverse) incision was made through the pregnant ewe’s abdominal wall to allow the obstetrician’s hand to manipulate the uterus into proper position for percutaneous fetal RV entrance. The 17-gauge cannula and trocar were introduced percutaneously in the ewe’s abdomen, through the uterine wall, and into the chest wall of the fetus under ultrasound guidance. Once in the body of the RV, the trocar was removed, and pulsatile blood return was confirmed. The wire-balloon-stent or TEHV combination was inserted through the cannula until the wire and then balloon with stent were seen by ultrasound to be crossing the pulmonary valve. In the later cases, as lessons were learned from the developing procedure, prophylactic epinephrine 10 µg/kg was given through the sidearm of the Touhy-Borst into the fetal heart through the lumen of the balloon catheter. When the stent was centered on the pulmonary valve annulus, the balloon was inflated with a commercially available inflation device to 4 atm. The balloon was deflated, and the balloon, wire, and cannula were removed from the ewe completely. The heart was observed for the development of pericardial effusion or bradycardia for approximately 45 min. Enlarging pericardial effusions were treated with needle drainage, and bradycardia was treated with intramuscular or intracardiac epinephrine and atropine. Procedural success was defined as implantation of the zinc stent or TEHV across the pulmonary valve annulus or in the main pulmonary artery (MPA).

**Data Analysis.** Biaxial mechanical testing and structural characterization data were compiled and analyzed using Tecplot (Tecplot, Bellvue, Washington). Hemodynamic assessment data was compiled and analyzed using MATLAB (The MathWorks, Natick, Massachusetts), as well as Matplotlib and DataGraph graphing software. Gel permeation chromatography data were compiled and analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, California). All data presented as mean ± SD.

**Results**

**Valve Structural Characterization.** The average tortuosity of the PCL valve material was 1.13 ± 0.11, indicating a moderate tortuosity level. This measurement is similar to previously reported values of engineered heart valve tissues (29). NOI for the PCL valve material was 20.7 ± 7.1%, suggesting slight alignment. A material with slight alignment would likely produce a mildly anisotropic response to equibiaxial strain. This mechanical response can be seen in the biaxial mechanical testing results. There was strong agreement in both tortuosity and NOI between the 2 magnifications, with 20.3% NOI and a tortuosity of 1.12 for the 500× samples and 21.1% NOI and a tortuosity of 1.13 for the 1,000× samples, indicating no dependence on SEM image magnification. The measured fiber density of the PCL valve material is 0.22 ± 0.09, indicating that the surface layers of the material cover roughly one-fourth of the total area. This measurement takes into account only the
topmost layers of the material, as opposed to all visible layers in a given SEM image.

**VALVE PROPERTIES: MECHANICAL TESTING.** Biaxial mechanical testing of the PCL valve material demonstrated a maximum stress of 1,357 ± 116 kPa in the primary (circumferential) direction and 1,036 ± 93 kPa in the secondary (radial) direction, giving a resulting PD/XD ratio of 1.32 ± 0.70, representing mild anisotropy with a stronger primary direction. Representative mechanical testing results can be seen in (Figure 3). Anisotropy is desired in engineered heart valve tissues to mimic the anisotropic responses of the circumferential and radial directions of the native valve leaflet (30).

**VALVE PROPERTIES: HEMODYNAMIC ASSESSMENT.** Figure 3 shows the flow rate, pulmonary artery pressure, and transvalvular PG waveforms. The mean PG obtained across the TEHV was 8.560 ± 0.139 mm Hg, with a peak of 20 mm Hg, and the EOA was 0.1000 ± 0.0007 cm². The RF was 2.35 ± 1.99%. Video 1 shows the opening and closing of the valve en face, and Figure 3 shows still frames of the open valve during acceleration, peak, and deceleration phases of the cardiac cycle and the closed valve in diastole. The pinwheeling index obtained with the TEHV was 0.404 ± 0.01.

**VALVE PROPERTIES: ACCELERATED DEGRADATION.** Accelerated degradation testing on the electrospun PCL valve material demonstrated an initial period of steady mass with a rapid decline in $M_w$ and $M_n$ and a corresponding increase in PDI. This initial period was followed by steady mass loss coupled with continued declines in $M_w$ and $M_n$ and a relatively stable PDI. By SEM, fiber fractures began to appear in the 60-min samples, while the 120-min samples demonstrated near-complete structural loss (Figure 4).
DEVELOPMENT OF TRANSCATHETER APPROACH.

Ten pregnant ewes with 16 fetuses were placed under general anesthesia for development of the percutaneous and fetal percutaneous and transcatheter TEHV procedure (Table 1). Procedures were not attempted on 2 fetuses (#3B, #3C), owing to poor fetal position for intervention.

**Zinc stent implantation.** In order to learn the percutaneous, transcatheter technique, a pilot study using 9 pregnant ewes (#1 to #9) was undertaken, during which zinc stents were implanted in the pulmonary annulus or MPA without a TEHV. Stent implantation was attempted in 12 fetuses and was successful in 5 fetuses. Four of these 5 fetuses had immediate bradycardia, leading to fetal demise, and 2 had an associated pericardial effusion. The only fetus to survive (#8) had the stent implanted in the MPA, instead of across the pulmonary annulus. This fetus survived 6 days and died due to maternal strangulation of bowel, as a result of a large laparotomy incision created for fetal positioning. The small, medial Pfannenstiel incision allowed the obstetrician’s hand to manipulate the fetus into optimal position through the thin uterine wall. Alternatively, a spinal needle through the ewe’s abdominal wall was often used to manipulate a fetal extremity away from the anterior chest wall. Success was also more common when

**FIGURE 4 In Vitro Degradation of Tissue-Engineered Heart Valve PCL**

(A) Electrospun polycaprolactone (PCL) sheets were subjected to accelerated degradation in sodium hydroxide, demonstrating an initial period of minor microstructural changes, followed by rapid structural collapse. (B) Mass loss over the accelerated degradation time showed a similar profile of minor and then steady mass loss over time. (C) Molecular analysis of the degraded samples demonstrated rapid changes in molecular size (Mn), molecular weight (Mw) and polydispersity index (PDI) at early time points, followed by steady decreases at later times.
prophylactic intracardiac epinephrine was administered immediately prior to inflating the balloon. The breed of ewe also may have played a role in survival. Last, minimizing fetal blood loss through the cannula, between the time that the trocar was removed and the balloon or stent were inserted, was critical for fetal survival.

**TEHV Implantation.** Using what was learned in the fetal stent pilot study, TEHV implantation was attempted in 2 fetuses (#9A, #10). In fetus #9A, the TEHV was implanted in the MPA, which resulted in obstructed blood flow to the branch pulmonary arteries. Immediate bradycardia was noted, which did not respond to epinephrine. In fetus #10, the TEHV was implanted in the pulmonary annulus. Laminar flow was seen across the TEHV without regurgitation (Figure 5). Subsequent imaging revealed that the stent had migrated to the MPA. The fetus survived the procedure and was born at term gestation. He was alive at 18 months of age.

Success in stent and TEHV implantation improved with time, with 5 of the 7 successful procedures occurring in fetuses #7B, #8, #9A, #9B, and #10. However, bleeding, bradycardia, pericardial effusion, and stent migration represent common serious complications associated with this procedure.

**DISCUSSION**

This multidisciplinary work shows the feasibility of replacing the fetal pulmonary valve with a percutaneous, transcatheter, fully biodegradable TEHV. Replacing a stenotic fetal valve with a functional TEHV has the potential to interrupt the development of single-ventricle heart disease by restoring proper flow through the heart. Prenatal treatment provides a potentially curative strategy, as opposed to postnatal palliation of single-ventricle disease, which is fraught with significant morbidity and mortality. Although the various components of the TEHV and the techniques and methods for catheter-based insertion will clearly benefit from further improvements and refinements, establishment of feasibility of this methodology serves as a foundation for future in vitro and in vivo evaluations.

In vitro work on the valve PCL material demonstrated that the designed and created valve was
Biodegradable, with a short period of stable mass followed by steady mass loss. Previous studies with similar electrospun PCL material suggest that this degradation is similar to what the valve leaflets will experience over 3 to 4 months in vivo (12). The zinc stent material used has previously been shown, in a rat aortic implantation model, to have no evidence of chronic inflammation, localized necrosis, or progressive intimal hyperplasia, and should retain mechanical integrity to 6 months (11). Although properties of the fetal milieu as an environment for tissue engineering remain to be elucidated, previous studies of tissue engineering topics have demonstrated fetal cells and tissue to have higher regenerative capacity and lower inflammatory response compared with adult tissues, suggesting that the time frames of degradation of these materials should allow for cellular infiltration and neotissue formation (31).

Biaxial mechanical testing demonstrated that the electrospun PCL was anisotropic, with a stronger circumferential direction. Native heart valve leaflets have been shown to be anisotropic in a similar manner, although the degree of anisotropy in native valves is much higher than that shown in our fetal valve design (32). The maximum stress of the PCL valves was measured at over 1,000 kPa, much higher than the maximum stress experienced by an adult aortic valve in vivo, suggesting that the PCL valve has a suitable strength for in vivo performance without failure (33). Comparison of the microstructural characteristics and mechanical behavior of the PCL material demonstrates the importance of hierarchical design in tissue engineering. The PCL fibers had a 20% alignment toward the circumferential direction, while the mechanical strength of the circumferential direction was 32% higher than the radial direction.

The hemodynamic performance of the TEHV was assessed in a dedicated right heart pulse duplicator. Although no true control valve of similar size is available for comparison, the peak PG reported for the TEHV was 20 mm Hg, while peak PGs reported in children receiving a Melody valve (Medtronic, Minneapolis, Minnesota) or SAPIEN valve (Edwards Lifesciences, Irvine, California) in pulmonary conduits were 13.5 mm Hg (by catheterization at time of implant) and 18.7 mm Hg (by echocardiogram at 1-month follow-up), respectively (34–36). Postoperative pressure gradients of surgical valves implanted in the pulmonary position in pediatric patients (determined by echocardiogram) were reported as 16 to 44 mm Hg (37). The EOA was found to be 35.4% of the available geometric area of the valve. For comparison, a 26-mm SAPIEN 3 aortic valve has been shown to have an EOA of 2.1 cm², roughly 39.6% of its available geometric area based on inflow annular diameter. The mean RF of 2.35% for the TEHV is consistent with trivial pulmonary regurgitation (38,39). The degree of pinwheeling of the TEHV (0.404 ± 0.01) was comparable to that obtained with SAPIEN 3 transcatheter aortic valves (0.122 to 0.366) and thus is indicative of promising leaflet durability (15). In summary, the in vitro evaluation revealed a hemodynamically competent and non-stenotic valve with predicted short-term durability similar to commercially available fixed-tissue transcatheter heart valves. Although the initial hemodynamic evaluation results are promising, further
hemodynamic evaluation at a range of physiological cardiac outputs and heart rates will further inform the future rapid design and development of the TEHV. In addition, future investigation combining the degradation techniques with the hemodynamic evaluations will aid in the understanding of how valve mechanics will change in vivo.

Although many TEHVs have been developed, a lack of mechanistic studies limits the scientific understanding of the mechanisms of neotissue development, as well as clinical success and failure (40). Transcatheter deployment of a tissue-engineered venous valve in a sheep model demonstrated successful function and endothelialization at 2 weeks post-deployment (41). In another study, transcatheter TEHVs, derived from porcine pericardium, were implanted in the subcutaneous tissue of rats and demonstrated limited inflammation and a loss of mechanical properties over the first few weeks post-implantation. These valves, however, were not tested in a functional biological position (42). Development of the large animal model presented in this work will allow for a deeper understanding of these biological mechanisms, as well as the critical design parameters needed to design an ideal TEHV, such as degradation profile and inflammatory status (40).

The bare-metal zinc stent deployments were used to learn the technique of percutaneous, transcatheter implantation into the fetal pulmonary valve annulus, prior to implanting TEHVs. The extremities of the fetal sheep, including the hooves, are typically covering the anterior chest wall access site, making the approach difficult. Manipulation of the extremity away from the chest with a needle percutaneously is often useful. In addition, the pregnant ewe abdomen is flat with the ewe supine, making the approach more difficult than in the human. Furthermore, the fetuses frequently lie under the maternal thighs, which adds complexity. A small Pfannenstiel incision allows the obstetrician to manipulate the fetus into proper position with her hand on the uterus, and this improved success. The valve is still implanted percutaneously through the ewe’s abdominal wall, even when the obstetrician is positioning the fetus through the uterine wall. The high rate of fetal death after stent implantation into the pulmonary valve annulus may be due to acute incompetence of the pulmonary valve.

The very low-profile bioabsorbable zinc stent allowed the use of a 17-gauge cannula for the delivery of the TEHV. As large as a 16-gauge needle has been used in human fetal pulmonary balloon valvuloplasty cases (6). Therefore, the smaller 17-gauge needle is an acceptable size for human fetal intervention.

Future work will aim to use this model to evaluate fetal TEHV performance, the development of neotissue development, and stent and valve degradation in vivo.

**STUDY LIMITATIONS.** As there are currently no large animal models of single-ventricle anomalies, all procedures were performed on healthy lambs with presumably normal heart valves. Owing to this, it is difficult to determine if the TEHV is capable of reversing the development of single-ventricle anomalies, as has been recently described in human patients after balloon valvuloplasty. However, the development of this transcatheter fetal technique may still provide many insights into the outcomes related to the stent and valve, as well as the mechanisms of TEHV neotissue development.

Although this pilot study demonstrates the feasibility of performing fetal valve replacement in utero with a TEHV using a percutaneous, catheter-based delivery system, both the prototype and methodology need substantial refinement before translation to the clinic. In fact, the prototype was created from several pre-existing devices which were assembled and used to demonstrate proof of principle rather than to serve as the first-generation product. Further refinement of methods to reduce or even eliminate the morbidity and mortality of in utero fetal cardiac puncture are needed. Significant morbidity and mortality from bleeding, arrhythmias, pericardial effusion, and premature delivery are all possible. In addition, substantial refinements of the biodegradable stent and TEHV scaffold are needed prior to beginning studies to elucidate the cellular and molecular mechanisms of valvular neotissue formation in the fetus, which will ultimately need to be ascertained in order to design and optimize this product. Although degradable stents have the benefit of having a finite life within the body, they also consequently lose mechanical integrity over time. The lifetime of the stent and its changing mechanical properties within the body are critical to the clinical performance of the stent, and stent degradation needs be investigated (43,44). Additionally, while the initial hemodynamic evaluation of the TEHV was promising in its basic function with trivial regurgitation, it is difficult to draw conclusions from measures of valve performance such as EOA and pinwheeling index, owing to the lack of a commercially available size-matched control. Further studies are necessary to assess valve function under a range of hemodynamic parameters and at different time points of degradation in order to inform future TEHV design and development.
Despite these challenges and the nascent stage of this technology, these data support the feasibility of performing percutaneous and transcatheter fetal heart valve replacement and hold extraordinary potential for revolutionizing the treatment and prevention of complex congenital cardiac anomalies.

CONCLUSIONS

In this work, we have reported the development of a fully bioresorbable valve and stent which can be used for pulmonary valve replacement in utero. We characterized the components of this system in vitro prior to implantation, including accelerated degradation, mechanical, and hemodynamic testing. A percutaneous and transcatheter deployment technique was developed to implant this tissue-engineered valve into a fetal lamb model. The multidisciplinary approach serves as a paradigm for the development of new technologies for translational regenerative medicine, combining the unique expertise of engineers, scientists, and medical specialists for the central goal of improving health outcomes.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: We created a fully biodegradable fetal valve and stent, using tissue engineering methods, that was deployed in the fetal ovine pulmonary annulus with a percutaneous and transcatheter approach. Valves were made of electrospun PCL and mounted on degradable zinc-aluminum alloy stents. They were analyzed in vitro through accelerated degradation, mechanical, and hemodynamic testing. The hemodynamic performance of the TEHV in a right heart pulse duplicator showed a peak pressure gradient of 20 mm Hg, which is comparable to gradients obtained in transcatheter heart valves placed in children. The valve had a regurgitant fraction indicative of only trivial regurgitation. The very low-profile valve was successfully implanted in a fetal lamb through a 17-gauge cannula percutaneously, and the fetus was delivered alive at term. This work showcased the importance of a large, multidisciplinary team in solving multifaceted medical problems. Development of our successful stent, valve, and delivery technique would not have been possible without the combined work of many clinicians, scientists, and engineers.

TRANSLATIONAL OUTLOOK: We showed the feasibility of implanting a fully biodegradable TEHV into a fetal large animal model using only a 17-gauge needle. This study laid the groundwork for investigating the regenerative capacity of the fetal milieu for neovalve formation. Future work will use this model to evaluate fetal TEHV performance, the development of neotissue development, and stent and valve degradation in vivo. Fetal TEHV implantation has the potential to interrupt the development of congenital heart disease in utero and to promote a biventricular circulation by replacing abnormal valves and improving flow dynamics. Such a low-profile biodegradable valve also has great potential for transcatheter implantation in infants, which is not possible with current technology.
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**KEY WORDS** congenital heart disease, tissue-engineered heart valve, transcatheter heart valve, translational medicine

**APPENDIX** For a supplemental video, please see the online version of this paper.
Approximately 9 in 1,000 infants are born with congenital heart defects, which are a significant contributor to early mortality and long-term morbidities (1). Current treatment of these defects includes invasive reconstructive surgeries and the use of bioprosthetic valves or valved conduits that can be delivered surgically or minimally invasively. Some fetal cardiac anomalies, including pulmonary atresia with an intact or almost intact ventricular septum, can lead to single ventricle disease. However, due to the use of foreign materials in their components (including synthetic materials or grafts), these implants can cause multiple adverse effects once implanted. Among these sequelae are calcification, contracture of the implant, immune rejection, and tissue degeneration. As a result, replacement of bioprostheses is often warranted, necessitating further intervention, often within 5 years after the initial placement (2).

Tissue engineered heart valves (TEHVs) have potential to circumvent the aforementioned issues caused by the use of synthetic materials and xenografts or homografts in bioprostheses. In this paradigm, biodegradable scaffolds are used to promote the host’s own cells to populate the implant and become dynamic tissue that has the capability to grow and remodel. In the fetus, regeneration and formation of new tissue are particularly effective, thus motivating fetal implantation of TEHVs as a favorable strategy for subsequent growth and adaptation.

In this issue of JACC: Basic to Translational Science, Zakko et al. (3), a highly interdisciplinary group of researchers have described the first transcatheter, fully biodegradable tissue engineered fetal heart valve. By using a degradable zinc-aluminum alloy for the stent frame, and electrospun polycaprolactone for the leaflets, they successfully manufactured a valve that could be crimped over a 17G trocar to ultimately allow percutaneous delivery. It is a notable feat of engineering to overcome the challenges associated with fabricating a functional biodegradable valve, and further designing it so that it can be crimped to such a small diameter and retain its functionality after percutaneous delivery. Current transcatheter technology was used to crimp the valve onto an angioplasty balloon for subsequent delivery through a catheter. Once at the valve annulus, the balloon can be inflated to expand the stent, and the polycaprolactone leaflets overtake the function of the stenotic valve.

Extensive in vitro testing was performed with the valve to validate its performance including measurements of stenosis and regurgitation, accelerated aging, cyclical mechanical testing, and dynamic flow loop evaluation. Notably, the valve showed negligible regurgitation when subjected to pulsatile flows and a peak transvalvular gradient of 20 mm Hg.

Remarkably, the researchers showed that the valve could be delivered percutaneously into the fetal pulmonary annulus in an ovine model.
implantation, echocardiographic imaging of the valve showed laminar flow with no stenosis or regurgitation. The fetus matured after valve implantation and was delivered at full term.

This success was not without challenges and limitations. The experiments revealed multiple challenges associated with the procedure. Overall, in 16 fetuses, only 1 fetus survived the implantation and was delivered at term. In a pilot study of 9 pregnant sheep with a total of 12 fetuses, the researchers attempted implantation of a zirc stent without any polycaprolactone leaflets to learn about the minimally invasive approach. Implantation was successful in 5 of 12, and 4 of these fetuses did not survive due to bradycardia or pericardial effusion. In the 1 successful implantation, the stent was implanted in the main pulmonary artery instead of the pulmonary annulus, and the fetus subsequently died due to a maternal strangulated bowel. The researchers identified multiple improvements in percutaneous delivery techniques from the pilot study including: 1) fluoroscopy of the abdomen in addition to ultrasound; 2) anterior right ventricular free wall puncture; 3) optimal fetal positioning; 4) a small medial Pfannenstiel incision on the contralateral side of the fetal position to allow manipulation of this position through the uterine wall; 5) administration of intracardiac epinephrine before balloon inflation; 6) careful choice of ovine breed; and 7) minimizing fetal blood loss through the introducing cannula. Guided by the new knowledge gleaned from the pilot study, subsequent implantations were more successful, but still revealed serious complications associated with this procedure including heart block, stent migration, bradycardia, and pericardial effusion. In the successful implantation, the valve demonstrated laminar flow without regurgitation but subsequently migrated to the main pulmonary artery. Still, the fetus survived to term and was alive at 18 months.

A major contribution of this paper is the development and percutaneous delivery of a fully biodegradable heart valve in the pulmonary annulus. This is the first report of transcatheter delivery of a TEHV. Previously, researchers (4) had used nitinol stent frames for stem-cell based pulmonary valves which do not grow with the child, and require a fetal thoracotomy for delivery through the right ventricle using a delivery system that is approximately 4 times larger than that described in this work. Indeed, percutaneous delivery has many advantages including obviating the need for maternal and fetal incisions and a lower incidence of iatrogenic preterm prelabor rupture of membranes (5).

Future work will be needed to investigate the cause of the many complications reported with this procedure, to optimize the design of the delivery system and the implant, and to fully characterize the degradation of the stent, and the rate of neotissue formation.

In summary, the work shows the first proof-of-concept of transcatheter fetal implantation of a TEHV which has transformative potential to alter fetal hemodynamics and prevent complex cardiac anomalies such as single ventricle disease.  

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**KEY WORDS** congenital cardiac anomalies, in utero delivery, tissue engineered heart valves, transcatheter delivery
TRANSLATIONAL TOOLBOX

Update to Drugs, Devices, and the FDA
How Recent Legislative Changes Have Impacted Approval of New Therapies

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SUMMARY

Two major legislative actions since 2015, the 21st Century Cures Act of 2016 and the U.S. Food and Drug Administration (FDA) Reauthorization Act of 2017, contain significant provisions that potentially streamline drug development times, and by extension, may reduce costs. Evidence suggests, however, that development times have already been significantly affected by previous legislation and FDA programs, through accelerated approval pathways and adoption of more flexible definitions of clinical evidence of efficacy. The COVID-19 pandemic is pushing researchers and commercial entities to further test the limits of drug and vaccine development times and approvals, at an as yet unknown level of risk to patients. COVID-19 drug and vaccine trials are even now making use of accelerated drug approval programs, blended trials, and adaptive trial design to accelerate approval of therapeutics in the pandemic.

After the Federal Food, Drug, and Cosmetics Act of 1938, all drugs marketed in the United States have been required to pass safety approval by the U.S. Food and Drug Administration (FDA) (1). The 1962 Kefauver-Harris Amendments to the act further required that drugs have proven efficacy for their intended use (2). However, the complex regulatory environment for approving safety and efficacy of new drugs and biologics (DABs) has been blamed for delays in both DAB development and deployment of critical therapies to patients in need, a progressive decline in annual drug approvals, and burgeoning costs of DAB development. Basic processes of FDA approval of new DABs were covered in a previous review in 2016 (3). Those are briefly summarized here, followed by a review of the changes in the drug development legislation in the last 5 years and their effects on the timeline of new DAB development, a followed by brief summary of some strategies being employed for DAB approval in the setting of the COVID-19 pandemic.

PATHWAYS TO DRUG APPROVAL

Following preclinical development, including in vitro and in vivo (animal) studies, and before proceeding to testing in any human subjects, the FDA must be involved in all drug development in the United States. There are 3 common pathways that developers can pursue (3).

In a standard pathway for common drugs, this starts when the researcher or sponsor (usually a commercial entity) files an Investigational New Drug (IND) application—either an Investigator IND or...
Commercial IND—that, once approved, will allow the investigational drug to be transported from the manufacturer to and among interstate research entities for clinical studies. The application includes information about the drug, the researcher’s qualifications, the manufacturing process, the clinical study protocols, and commitments to obtain informed consent and institutional review board approval. If after 30 days from filing there is no objection from the FDA, clinical studies can commence in what is generally divided into 3 phases of testing, progressing from small studies in healthy volunteers to large studies in targeted patient populations. Timelines, characteristics, and success rates of the standard clinical phases are summarized in Figure 1.

Before progressing from either Phase I or II, the investigator must pause and provide information on the safety of the new drug, any new information discovered in each of those phases, and any changes in manufacturing or drug preparation. If there is no objection from the FDA at each of these stages, the drug can proceed to the next clinical phase. Upon completion of successful Phase III studies, a process that takes around 8 to 10 years for most common drugs, the investigator or sponsor pays an application fee and files a New Drug Application (NDA) that includes extensive information on the drug, the results of all phases of testing, manufacturing and facilities, quality control, labeling, and risk evaluation and mitigation. For an NDA, the FDA requires “substantial evidence” of a drug’s safety and efficacy, a requirement that until recently was interpreted to mean at least 2 adequate and well-controlled Phase III trials with convincing demonstration of efficacy (4). The FDA has 60 days to file the application, unless it raises questions, and after the filing, it must review the application within 180 days. Once the FDA review has occurred, if there are no objections, the manufacturer can make and market the drug for its approved clinical use.

A second pathway to drug approval is available when an emergency situation does not permit sufficient time for a standard IND process or institutional review board approval. These generally involve individual patients who have a serious or life-threatening illness for which delay of clinical treatment would be devastating, or for which no treatment protocols exist. The treating physician can apply for an Emergency IND by directly contacting the FDA, which if immediate therapy is needed, can approve such applications over the phone. For less emergent conditions, a 30-day FDA review period applies (5). The investigator can proceed with treatment, but must still complete a full IND in a timely fashion.

In a third pathway, a Treatment IND can be sought for approval for use of an experimental drug that is showing promise in clinical studies, but has not completed them. These are also termed “Expanded Use INDs” (3). These can be issued for a drug that is already under investigation or has completed clinical studies and is awaiting an NDA approval that the sponsor is actively pursuing. The drug must be intended to treat a serious or life-threatening condition for which there is no satisfactory alternative treatment (6). Treatment INDs are often used to bridge the period of time between Phase III efficacy demonstration and NDA approval for those patients who are trial participants and who have benefitted from the drug, but they can also be used to allow treatment of nonstudy patients and populations.

Two other pathways for approval exist to be used only under very extraordinary circumstances. In some situations, such as a nuclear accident or terrorism attack involving a biological or radiation weapon, DABs can be approved and deployed in the absence of human trials altogether—primarily because to carry out human trials that, required, for example, exposure to lethal doses of radiation, would be unethical (7). Additionally, in the face of an officially declared public health emergency, the FDA can provide Emergency Use Authorizations (EUAs) (8) that permit public release of the DAB after Phase II efficacy has been shown. Phase III human clinical studies are deferred, and once the emergency has abated, confirmatory studies must be carried out after market approval (they are then usually termed Phase 4 studies). This latter “pathway” is of particular interest in the COVID-19 pandemic.

**Recent Legislation Affecting FDA Approvals**

Between 2015 and the present, 2 major legislative initiatives address long DAB development times: the 21st Century Cures Act (9), and the FDA Reauthorization Act (FDARA) of 2017 (10), part of which contains the sixth revision of the Prescription Drug User Fee Act (PDUFA VI). Most of the provisions in these laws do not change the basic steps in drug development reviewed previously, but concentrate on FDA funding and staffing to facilitate FDA review;
establish programs in which the FDA works more closely with the DAB development entity to reduce reiterative reviews and delays; and encourage reinterpretation of existing FDA rules to allow more innovative clinical trial design and use of biomarkers and “real-world” data in evaluating efficacy. Basic provisions of these legislative efforts are summarized in Table 1 (9–11).

CHANGING DAB DEVELOPMENT TIMES

Driven largely by budget limitations that prevented the FDA from employing sufficient personnel to provide timely processing of NDAs, FDA review times ran around 33 months in 1987 (12). In the face of a growing AIDS crisis, the pharmaceutical industry offered funding to the FDA in the form of “user fees” in
TABLE 1 Relevant Features of Major U.S. Drug Development Legislation, 2015 to 2019

| Year      | Legislation                          | Provisions                                                                 |
|-----------|--------------------------------------|-----------------------------------------------------------------------------|
| 2016      | 21st Century Cures Act               | - Continued to build on previous legislation to incorporate patient perspectives into drug and device development, and to modernize clinical trials design through use of real-world evidence and clinical outcomes assessments.  
- Provided for recruitment and retention of appropriate experts, and established 2 new expedited product development programs: 1) RMAT; and 2) breakthrough devices.  
- Directed the FDA to create intercenter institutes to coordinate activities in major disease areas between the centers for drug, biologics and device centers, and to improve the regulation of combination products. |
| 2017      | FDARA of 2017                         | - Continued the 5-yr reauthorization cycle of human medical product user fees (PDUFA VI—see following text), and allows the FDA to use the revenue to support the marketing applications for brand-name and generic drugs, biological, and biosimilar products, and medical devices.  
- Established the renewed fees related to drugs, devices, generic drugs, and biosimilars (PDUFA)  
- Reauthorized existing programs such as the Orphan Drug Program  
- Included the RACE for Children Act, requiring evaluation for new molecular targets and biologics intended for adults that are specifically targeted for cancers in children, elimination of orphan exemption from pediatric studies  
- Established rules for device inspections, established means to improve access to generic drugs, and included provisions for FDA performance reporting, as well as other administrative enhancements |
| 2017      | PDUFA VI (a section of the FDARA)     | - Renewed drug development user fees  
- Provided for system enhancements, such as improvement of the electronic submissions system  
- Required the FDA to work toward incorporating adaptive, Bayesian, and other complex trial designs and explore use of real-world evidence in approval processes  
- Required the FDA to work on defining biomarkers, trial endpoints, and other drug development tools  
- Required the FDA to provide means to incorporate patient and care provider perspectives into clinical trial designs  
- Provided for enhanced financial and staffing transparency |

Summarized from Dabrowska and Thaul (4), Van Norman (5), and U.S. Food and Drug Administration (6).

FD – U.S. Food and Drug Administration; FDARA – Food and Drug Administration Reauthorization Act; PDUFA – Prescription Drug User Fee Act; RACE – Research to Accelerate Cures and Equity; RMAT – Regenerative Medicine Advanced Therapy.

exchange for commitments to accelerate reviews, with the intention that these fees would in large part be used to hire sufficient staffing to accomplish this goal (13). The proposed fees caused great discomfort among many policy makers, because it would create a dependence for essential funding by the FDA on the pharmaceutical companies it regulates. Nevertheless, user fees were adopted and have remained an important funding source for FDA reviews, providing about $1.22 billion (79%) of the FDA’s regulatory spending for DABs in 2017 (14). The PDUFA required that these funds only be used to expedite reviews of human DAB applications. Although the PDUFA included a 5-year sunset provision, it was so successful (the FDA reviewed 90% of DAB applications within 6 or 12 months after submission) (13), Congress reauthorized the PDUFA in successive years (PDUFA II to VI) requiring further reductions in review times. Subsequent provisions authorized the use of user fees to shorten clinical trial times, funded the FDA program for evaluating post-market DAB safety—a provision that was later removed—and reduced the number of review cycles for DAB approvals. The PDUFA section of the FDARA of 2017 raised these user fees and provided for annual increases through 2022. The FDARA also imposed new fees for generic drug applications ($171,823), generic drug manufacturing facilities ($211,087 annually), medical device pre-market notifications ($10,566), pre-market authorization submission ($310,764), and biosimilar applications ($1,746,745) (13). In 2020, the NDA user fee is $2,942,965 (15), compared with the original user fee of $100,000 in 1992 (13). The FDARA sets the goals for complete reviews of 90% of all applications within 6 to 10 months for biosimilars, 8 to 10 months for generic drug applications, and 180 days for medical devices (16). Under the FDA’s PDUFA VI commitment letter, the FDA agreed to undertake initiatives to explore the use of “real-world data,” hire new staff to support increased use of biomarker and surrogate endpoints, and promote use of adaptive, Bayesian, and other novel clinical trial designs (14,17). DABs that meet an important public health need will be fast-tracked with an aim for the FDA to act at least 1 month before the standard PDUFA deadline.

As a result of the FDARA, user fees have risen faster than the FDA budget as a whole, and now make up 75% of the scientific review budgets for brand name and generic drugs, and over 40% of the total 2016 FDA budget. The FDA’s dependence on industry funding has put pressure on Congress to maintain the FDARA to avoid substantial FDA layoffs (estimated as up to 5,000 full-time positions) (13). Many observers express concern that there has been a weakening of the...
regulatory independence of the FDA, and that this may have a substantial impact on the FDA’s efficacy and safety standards. Such issues call to mind the industry/regulatory interdependence between the Federal Aviation Administration and the airline industry/regulatory interdependence between the NASA and the Boeing 737.

Under the PDUFA, total review times at the FDA decreased from 1.2 years for the period 2006 to 2017, to 10.1 months in 2018 for a standard application and 7.6 months for a priority application (i.e., DABs that met FDA criteria as a therapeutic advance and received priority attention) (14). In 2017, Hwang et al. (19) reported that novel therapeutics enrolled in at least 1 accelerated development program at the FDA from 2012 to 2016 experienced decreased median development time of nearly 1 year compared with nonaccelerated therapeutics (7.1 vs. 8.0 years, respectively). DABs with Breakthrough Therapy (BT) designation (i.e., new therapies representing a substantial improvement over existing therapies for a serious or life-threatening condition) shaved over 3 years of development time compared with non-BT drugs (4.8 vs. 8.0 years, respectively) (19). However, despite this decline in review times, the total time for IND effective date to final approval appears to have increased in the last 12 years, from an average of 7.0 years for the period of 1997 to 2007, to 9.1 years from 2008 to 2017, due in large part to increased trial times that offset reduced FDA review times (14).

**Evolving Interpretation of FDA Requirements**

**CONTROLLED TRIALS.** The overall roadmap for pre-approval clinical drug testing at the FDA is largely unchanged over the last several decades, even by recent legislation. Current law requires that efficacy claims in the NDA be supported by “adequate and well-controlled trials” (4,20); however, under various legislative actions, including those in the last 5 years, what constitutes “adequate and well-controlled” is being interpreted with increasing flexibility. Although at least 2 adequate, controlled trials were originally required by FDA regulations, later legislation encouraged the FDA to accept a single pivotal trial under some circumstances—such as when data from other populations than the target 1 provide supportive evidence. From 2015 to 2017, the proportion of NDAs that included at least 2 controlled trials decreased from the previous level of 80% to 52.8%. Furthermore, the number of NDAs approved during that period that relied on at least 1 Phase III study using an active comparator rather than historic controls or placebo fell from 44% to 29%, and the proportion of NDA approvals based on randomized, uncontrolled studies increased from 4% to 17%. Despite these changes, however, the actual length of clinical trials themselves for NDAs increased from 2015 to 2017, with almost one-half (46%) including at least 1 pivotal trial of 6 months’ duration or more, compared with just 26% from 1995 to 1997 (21).

| Designation                      | Year/Legislation          | Criteria                                                                 |
|----------------------------------|---------------------------|--------------------------------------------------------------------------|
| Accelerated Approval             | FDA instituted in 1992, but legislation in 2012 (FDASIA) allowed use of surrogate endpoints. | DAB that treats a serious condition that fills an unmet medical need can be approved on the basis of a surrogate endpoint: for example, lab marker, radiographic images, physical sign, or other finding that is thought to predict clinical benefit. Surrogate or intermediate clinical endpoints are allowed. |
| Priority Review                  | 1992 PDUFA                | DAB that would be a significant improvement in the safety or efficacy of the treatment, diagnosis, or prevention of serious conditions when compared to standard therapy. |
| Fast Track                       | FDA Modernization Act of 1997 | DABs that treat a serious condition must fulfill an unmet medical need or provide therapy that is substantially better in safety and efficacy than existing ones. Fast-Track DABs may also be eligible for Accelerated Approval and Priority Review if criteria are met. |
| Breakthrough Therapy             | 2012 FDASIA               | DABs that are intended to treat a serious condition, and preliminary clinical evidence indicates that it may demonstrated substantial improvement over available therapy on a clinically significant endpoint. Surrogate endpoints allowed. |
| Regenerative Medicine Advanced Therapy | 21st Century Cures Act, 2016 | Regenerative medicine advanced therapy (cell therapy therapeutic tissue engineering product, human cell and tissue product, or any combination product using these) used to treat, modify, reverse, or cure a serious or life-threatening condition, and preliminary clinical evidence indicates that it has the potential to address an unmet medical need. |

*The FDA defines a serious condition as one that will have an impact on day-to-day survival, functioning or the likelihood that if left untreated a condition will progress from a less serious one to a serious one.

DAB = Drugs and Biologics; FDASIA = U.S. Food and Drug Administration Safety Innovations Act; other abbreviations as in Table 1.
TABLE 3 Drug Approval Times in Years in FDA Accelerated Pathways

| Track Designation | Major Cancer | Minor Cancer | Decrease in Review Time |
|-------------------|--------------|--------------|-------------------------|
| Fast Track        | 7.2          | 9.2          | 2.0                     |
| Breakthrough Therapy | 6.4       | 9.6          | 3.2                     |
| Accelerated Approval | 6.2       | 9.6          | 3.4                     |
| Priority Review   | 8.0          | 10.2         | 2.2                     |

Average times in years from Investigational New Drug application to U.S. Food and Drug Administration (FDA) drug approval for anticancer drugs and biologicals achieving accelerated track designation at the FDA from 2012 to 2017 compared with overall anticancer drug approval times for the same period (median of 8.3 ys, n = 115 drugs), and separated by categorization as a drug for “minor” versus “major” cancer. (7,4). “Regenerative Medicine Advanced Therapy designation was created too recently to comment on review times.

DRUGS FOR RARE DISEASES. The FDA has for some time now also emphasized flexibility in its approval criteria with respect to treatments of rare diseases. This has far-reaching implications in a new era of precision medicine in which subtypes of common conditions, such as cancers, can be defined by genotyping and pharmacogenomics. Such genetic conditions qualify as rare diseases in regulatory language if they affect fewer than 200,000 cases in the United States, and qualify under the Orphan Drug Act of 1983 for more flexible FDA testing standards, research grants, tax benefits, and 7 years of nonpatent exclusivity (22,23). DAB development for rare diseases is also able to access specific FDA pathways for approval.

ACCELERATED PATHWAYS AND DAB DESIGNATIONS. The FDA has instituted several designations to facilitate the development and review cycles of new drugs that meet an unmet medical need in treating serious or life-threatening conditions, all but 1 of which were legislated before 2012 (Table 2) (14,24,25).

The Orphan Drug Act of 1983 was followed by the Accelerated Approval (AA) program in 1992 that allows approval on the basis of surrogate endpoints that are seen as “reasonably likely” to predict clinical benefit, rather than demonstration of improvement of clinical endpoints per se. Completion of post-approval studies to verify the clinical benefit (Phase 4 confirmatory trials) is required to maintain market approval (25). “Fast Track” designation authorized by the FDA Modernization Act of 1997 allowed more frequent reviews with the FDA and expedited rolling reviews (26). DABs that are fast-tracked are also eligible for AA and Priority Review designations, which commits the FDA to act on an NDA within 6 months (compared with the standard review of 10 months) (27). The BT program essentially formalized FDA review processes in the AA program. FDA guidance then indicated that BT drugs might be approved on the basis of studies with alternative clinical designs that could be smaller in number of subjects and scope, and use surrogate endpoints or biomarkers to determine efficacy (28,29). The 21st Century Cures Act mandated that the FDA maximize use of these existing programs, including the use of alternative measures, such as radiographic imaging and biomarkers, as determinants of therapeutic efficacy rather than clinical outcomes alone (9). In addition, the 21st Century Cures Act authorized a new drug designation, the Regenerative Medicine Advanced Therapies (RMAT) designation, that also qualifies for accelerated approval pathways.

Drugs carrying an Orphan Drug designation can access these accelerated pathways, requiring smaller trials (median participants n = 96 vs. 290 for common diseases), avoiding randomization or double-blinding (30% vs. 80% and 4% vs. 33%, respectively, compared with trials for common diseases), and achieving approval on the basis of interim effects (e.g., disease response) rather than mortality/survival clinical endpoints. Between 2008 and 2018, the proportion of DABs approved under the Orphan Drug Act increased to 22% from a previous level of 18% (30). The number of DABs that qualified for FT designation tripled between 1989 and 2018, with most of that increase occurring between 2009 and 2018. Over one-quarter of new DABs approved between 2014 and 2018 were granted BT designation (31).

Too few new cardiovascular drugs have been approved in the last 5 years to comment with confidence about changing drug approval times, but increased utilization of accelerated pathways has occurred in other drug categories, particularly oncology. Around 95% of all new anticancer DABs approved between 2012 and 2017 in the United States used 1 or more of these expedited programs (32). These changes decreased development times significantly (4.8 vs. 8 years) (19). Yamashita et al. (24) found that for anticancer therapies, pursuit of any of the expedited programs was associated with reduced review times (Table 3), by a range of 2.0 to 3.4 years. This finding is similar to a previous study by Hwang et al. (32). Most DAB approvals that they examined in the AA program (88%) used a noncomparative study design (24). As more and more diseases are genetically subclassified, more drugs qualify for AA tracts. Almost two-thirds of all INDs (64%) now qualify for such programs (14).

The RMAT drug classification went live in March of 2017 and is too new to determine whether it has been associated with reduced development timelines (33). IS PATIENT SAFETY BEING COMPROMISED? Accelerated drug approval pathways shave time off of the approval process and may theoretically reduce DAB
development costs, but they raise concerns of whether, in the interest of saving time and money, sacrifices are made in patient safety. One study of approvals of BT and non-BT approvals of cancer drugs failed to show differences in response rates, new mechanisms of action, mortality, or serious side effects (32), suggesting that BT designation may not be accompanied by greater efficacy, and may not present increased immediate risks to patients. Another study, however, found that BT and AA approvals were associated with fewer randomized controlled trials in the approval phase, and that entities without supporting randomized controlled trials were significantly more likely to be associated with post-approval modifications in common adverse events (71% vs. 29%) and had higher odds of post-approval major modifications in warnings and precautions (88% vs. 62%) (34), highlighting the need for clinician vigilance when prescribing an FDA-approved treatment that is a graduate from an accelerated pathway (35).

HOW REGULATORY CHANGES HAVE IMPACTED THE DEVELOPMENT OF DABS IN THE COVID-19 PANDEMIC

Efforts to reach accelerated approval of drug treatments and preventative vaccines in fighting the COVID-19 pandemic have included substantial use of accelerated pathways to FDA approval. COVID-19 was declared a public emergency by the Secretary of Health and Human Services on February 4th, 2020, who also confirmed that circumstances existed to justify EUAs for drugs and biological products (8,36). EUAs can be issued by the FDA very quickly: the regulations call for an automatic authorization if the FDA does not object within 30 days of application.

As of May 11, 2020, EUAs have authorized the use of hydroxychloroquine and chloroquine, convalescent plasma, hyperimmune globulin, remdesivir, and fresenius propoven (propofol) 2% in the treatment of COVID-19 (37). Although some of these therapies represent new advances, the EUAs also approve new uses for already established drugs—under an EUA, a new use for a therapeutic agent is not considered unapproved use of drugs off label (38). One of the most controversial examples has been the EUA for hydroxychloroquine for treatment of COVID-19 infections. Hydroxychloroquine is an approved therapy for malaria, rheumatoid arthritis, and lupus, with significant toxicity and as yet unproven efficacy against COVID-19 (39). Hydroxychloroquine received FDA approval for treatment of COVID-19 infections via EUA despite very limited and controversial clinical evidence (40). On June 15th, 2020, the FDA revoked its EUA for hydroxychloroquine due to lack of efficacy.

The U.S. “Operation Warp Speed Vaccine Initiative”—a public-private partnership among government agencies and private entities to produce a vaccine by early 2021 (40)—will clearly take advantage of many of the established accelerated pathways, (e.g., AA, Priority Review, and BT designations). In addition, the indirect effects of PDUFA VI funding for FDA staffing and other resource needs are likely to be of help. Provisions of both the 21st Century Cures Act and FDARA require the FDA to encourage the development of innovative trial design, the use of Bayesian adaptive trials and blended trials, the use of surrogate endpoints, and the incorporation of real-world data in determining efficacy of therapeutics (9,10,41). Examples of entities that have announced studies employing such strategies include Johnson and Johnson’s (New Brunswick, New Jersey) vaccine trial (1 of 5 candidate vaccines supported by Operation Warp Speed), which announced on June 10th, 2020, that it would begin a Phase I/II study (42). Oxford University has also indicated that trials of their vaccine will proceed along a process blending Phases I and II, and will certainly be applying for EUAs if efficacy is shown (43). With reportedly around 100 companies and academic institutions competing to develop a COVID-19 vaccine (44), it is a virtual given that they will all try to make full use of the innovative trial designs and accelerated programs combined with early EUAs to race for approval.

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**KEY WORDS** COVID-19, drug approval, drug legislation, emergency use, expanded access, pandemic, vaccine approval
Large Animal Models of Heart Failure: A Translational Bridge to Clinical Success

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HIGHLIGHTS

- Preclinical large animal models play a critical and expanding role in translating basic science findings to the development and clinical approval of novel cardiovascular therapeutics.
- This state-of-the-art review outlines existing methodologies and physiological phenotypes of several HF models developed in large animals. A comprehensive list of porcine, ovine, and canine models of disease are presented, and the translational importance of these studies to clinical success is highlighted through a brief overview of recent devices approved by the FDA alongside associated clinical trials and preclinical animal reports.
- Increasing the use of large animal models of HF holds significant potential for identifying new mechanisms underlying this disease and providing valuable information regarding the safety and efficacy of new therapies, thus, improving physiological and economical translation of animal research to the successful treatment of human HF.

SUMMARY

Preclinical large animal models of heart failure (HF) play a critical and expanding role in translating basic science findings to the development and clinical approval of novel therapeutics and devices. The complex combination of cardiovascular events and risk factors leading to HF has proved challenging for the development of new treatments for these patients. This state-of-the-art review presents historical and recent studies in porcine, ovine, and canine models of HF and outlines existing methodologies and physiological phenotypes. The translational importance of large animal studies to clinical success is also highlighted with an overview of recent devices approved by the Food and Drug Administration, together with preclinical HF animal studies used to aid both development and safety and/or efficacy testing. Increasing the use of large animal models of HF holds significant potential for identifying the novel mechanisms underlying the clinical condition and to improving physiological and economical translation of animal research to successfully treat human HF.

The complexity of heart failure (HF) has challenged the scientific community for decades. Multifaceted signatures of pathophysiological mechanisms driving HF are under intense investigation. However, the heterogeneous nature of the disease has limited therapeutic advances in the field. Not surprisingly, the prevalence of HF continues to increase at an alarming rate. Currently, it is estimated that 6.5 million people in United States have HF; by 2030, HF will affect >8 million people (i). In addition,

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HF negatively affects the economy, costing several billions of dollars each year ($70 billion by 2030).

A defining characteristic of HF is the inability of the heart to pump enough blood to the body, which leads to poor quality of life for patients with this condition. In the past 30 years, the diagnosis of HF has evolved 2 primary categories: 1) HF with reduced ejection fraction (HFrEF), characterized by a resting ejection fraction (EF) of $\leq 40\%$ and traditionally referred to as systolic HF; and 2) HF with preserved ejection fraction (HfEF), characterized by a resting EF of $\geq 50\%$ and traditionally referred to as diastolic HF (2,3). Recently, a third category of HF was introduced to the field, referred to as HF with midrange EF, characterized by a resting EF range from 40% to 50% (4).

The combination of numerous risk factors (physical inactivity), comorbidities (obesity, hypertension, type 2 diabetes, chronic kidney disease), and disease modifiers (age, sex) associated with HF has made improving therapeutic options for treating the overall syndrome difficult. Contributing to these difficulties is the lack of ideal animal models that reliably replicate most of the pathophysiological features often found in human HF. Large animal models of HF (e.g., pigs, sheep, etc.) have some advantages in terms of clinical translation given key determinants of myocardial work and energy consumption, such as left ventricular (LV) wall tension, heart rate, and vascular wall-to-lumen ratios are more similar to humans (5-13). Thus, it could be argued that the use of preclinical large animal models of HF for the discovery of novel mechanisms underlying the syndrome and development and/or testing of new therapeutic options for the treatment of HF are not only warranted, but necessary to advance our understanding of this highly prevalent cardiovascular disease.

This state-of-the-art review outlines existing methodologies and physiological phenotypes of several HF models developed in large animals (Central Illustration). Historical and recent studies of HF in porcine, ovine, and canine models of disease are presented in Table 1. The translational importance of large animal studies to clinical success is also highlighted, providing a brief overview of recent devices approved by the U.S. Food and Drug Administration (FDA) alongside their associated clinical trials and preclinical animal studies.

**HF INDUCED BY PRESSURE OVERLOAD**

Chronic pressure overload resulting from aortic valve stenosis or systemic hypertension may ultimately lead to HF (14,15). Over time, sustained increases in myocardial work required to overcome chronic elevations in afterload can induce structural, physiological, and molecular changes that result in pathological cardiac remodeling (16-18). In addition, vascular dysfunction in numerous organs, including the heart, brain, skeletal muscle, and renal systems, are negatively affected and may further contribute to cardiovascular dysfunction. To maintain normal function (often measured as EF), the heart transitions to a compensated stage characterized by concentric LV hypertrophy and increased myocardial stiffness associated with decreased myocardial relaxation, increased LV filling pressure, pulmonary congestion, and decreased cardiac reserve (16,17,19,20). Patients who have transitioned to a compensated stage of function often show signs and symptoms similar to those observed in HFpEF.

Given the significant number of HF patients with antecedent hypertension and/or aortic stenosis, numerous large animal models of pressure overload–induced HF have been developed to enhance our understanding of how these pathological mechanisms contribute to disease development. Many of these models have incorporated parallel comorbidities, such as obesity, type 2 diabetes, and chronic kidney disease, into the overall design in an effort to more comprehensively imitate the clinical syndrome, alongside traditional physiological features of HF (e.g., pulmonary congestion, dyspnea, and exercise intolerance). As a result, animal models of experimental pressure overload–induced HF have been developed using surgical techniques such as transthoracic aortic constriction (i.e., aortic banding), renal wrapping, and renal microembolization in pigs, sheep, and dogs. Endocrine-mediated methods based on high-salt diets like deoxycorticosterone acetate have also been used. Transthoracic aortic constriction methods attempt to recreate aortic stenosis by narrowing the aorta, which results in both local increases in myocardial afterload and neurohumoral involvement; the severity of each depends on the location of the aortic constriction (e.g., ascending aorta vs. the descending aorta). Aortic banding increases the LV aortic pressure gradient, induces concentric LV hypertrophy, increases myocardial stiffness, and impairs myocardial relaxation similar to that observed in aortic stenosis. However, aortic banding fails to recapitulate calcification and fibrotic lesions in the
aortic valve or significant increases in vascular stiffness occurring along the length of the aorta as often seen in human aortic stenosis. Renal wrapping, renal microembolization, and implantation of deoxycorticosterone acetate pellets induce systemic hypertension via neurohumoral activation. Although effective, these methods are limited by their inability to incorporate genetic factors that often contribute to developing hypertension and use supra-physiological doses of salt that may also have disproportionate impacts on neurogenic and neurohormonal activation. With these general strengths and weaknesses in mind, the following sections discuss existing large animal models of pressure overload–induced HF and...
highlight the physiological and molecular phenotypes associated with each. 

**AORTIC BANDING MODELS.** Several different studies have examined aortic banding in swine in the absence of comorbidities. Cardiac pressure overload was induced by constricting the ascending aorta in 45-day-old Yorkshire pigs using a 60- to 70-mm Hg systolic pressure gradient over 2 months (21–23). Traditional experimental signs of HF in these animals included peritoneal ascites in the range of 100 to 2,000 ml in less than one-half of all aortic-banded animals. Signs of both LV and right ventricular (RV) hypertrophy were observed in parallel with diastolic dysfunction evident as increased end-diastolic pressure (EDP) depending on the severity of disease. This model demonstrated significant impairments to myocardial oxidative and high-energy phosphate bioenergetics measured using primarily nuclear magnetic resonance spectroscopy techniques.

Aortic banding was also used to induce chronic pressure overload induced HF in both 3- and 8-month-old Yucatan miniature swine using a 50- or 70-mm Hg systolic pressure gradient, respectively, placed on the ascending aorta over 6 months (see Figure 1 for surgical visualization) (24–32). In this model, classic signs of experimental HF included increased LV brain natriuretic peptide mRNA levels and lung weight. Molecular and physiological phenotypes were most reminiscent of HFPpEF in these animals, including global concentric hypertrophy, normal resting EF, diastolic dysfunction (increased end-diastolic pressure–volume relationship, impaired diastolic strain during both early and late diastole, altered cardiomyocyte calcium handling), increased fibrosis and altered regulation of the extracellular matrix (ECM), mitochondrial dysfunction, and significant sex-based disparities in disease manifestation. This model also exhibited signs of significant vascular dysfunction in both coronary and peripheral vascular beds, including the brain, in which significant cerebrovascular impairment was observed alongside cardiogenic dementia.

Other models of HF developed by using aortic banding in pigs included studies by Ishikawa et al. (33) and Yarbrough et al. (34). A customized rubber band with a fixed inner radius of 12 cm was placed on the ascending aorta of Yorkshire pigs (10 to 13 kg) that were subsequently followed for 3 to 5 months by Ishikawa et al. (33). These animals did not show historical experimental signs of HF, but did demonstrate preserved EF, diastolic dysfunction (increased end-diastolic pressure–volume relationship and increased EDP with pacing), and LV hypertrophy with increased fibrosis. Using an inflatable cuff placed on the ascending aorta of Yorkshire pigs, Yarbrough et al. (34) progressively narrowed the ascending aorta by inflating the cuff weekly over 5 weeks. Final measurements showed a pressure gradient of 66 mm Hg with diastolic dysfunction (increased LV EDP and Tau) and increased fibrosis associated with regional myocardial stiffness and altered levels of ECM regulatory biomarkers (MMP-7 and -14, TIMP-1 and -4). This more acute model of myocardial pressure overload also did not show traditional indicators of HF.

A more recent attempt investigated the heterogeneous aspects of HF by including comorbidities via a combination of Western diet (10 months) and chronic pressure overload using aortic banding (6 months; 70-mm Hg systolic pressure gradient) in female Ossabaw swine (35), a unique translational large

| Model of Heart Failure | LVEF | Type of Animal (Ref. #) |
|------------------------|------|------------------------|
| Pressure overload      |      |                        |
| Aortic banding         | Preserved or reduced Pig (27-35) |
|                        | Reduced Sheep (47-52) |
| Renal wrapping or embolization | Preserved Dog (59-64); pig (65) |
| Myocardial infarction  | Reduced Dog (86); pig (87,88,111); sheep (82-92) |
| Permanent coronary occlusion | Reduced Pig (93-100); sheep (89,101,102) |
| Coronary microembolization | Reduced Dog (103,104); pig (105-107); sheep (108-110) |
| Arrhythmia             | Reduced Dog (118,119); pig (120,121,124); sheep (122,123) |
| Pacing-induced tachycardia | Preserved or reduced Dog (125,127); pig (125,126); sheep (128) |

DOCA = deoxycorticosterone acetate; HF = heart failure; LVEF = left ventricular ejection fraction.
animal model genetically predisposed to obesity and metabolic derangement that does not develop HF from dietary intervention alone (36–43). Recently listed as a multihit model useful for examining the heterogenous nature of HFpEF by the National Heart, Lung, and Blood Institute HFpEF working group (44), these animals displayed classic experimental markers of HF, including increased lung weight and genetic signatures that indicated the induction of numerous HF-related genes (e.g., natriuretic peptides). Significant inflammation and metabolic derangement (obesity, insulin resistance, dyslipidemia) was observed at both clinical and molecular levels, which has been considered a major causative component of HFpEF (45,46). Molecular and physiological phenotypes were also evocative of HFpEF, including concentric LV hypertrophy, normal EF, diastolic dysfunction (increased end-diastolic pressure–volume relationship, impaired diastolic strain during both early and late diastole, titin isoform shift, altered cardiomyocyte calcium handling), changes in the composition of ECM, and mitochondrial dysfunction. Biomarkers with potential relevance to HFpEF (e.g., Pentraxin-3 and interleukin 1 receptor-like 1) were also observed in parallel with significant microvascular dysfunction in coronary and peripheral (skeletal muscle, brain) vascular beds (35).

In contrast to swine models, the development of systolic dysfunction tends to precede diastolic dysfunction in ovine models of pressure overload–induced HF. Aortic banding of the ascending aorta in sheep was accomplished using a number of different methods, varying from static banding in young animals (47) to adjustable inflatable occluders in adult animals age 6 months to 2 years (47–52), which resulted in a wide range of systolic pressure gradients measuring 25 to 80 mm Hg. Demonstration of experimental HF was typically presented as a decrease in fractional shortening or EF, which indicated pressure overload in sheep might be more representative of HFrEF. Two of these studies addressed the often asked but understudied question, “What happens to the heart if the cardiac pressure overload is removed?” (49,52). These studies demonstrated some plasticity for the myocardium to return to normal via reversal of pathological cardiac remodeling, numerous cellular markers of apoptosis, and ECM regulation and modification.

Acute and chronic models of canine pressure overload-induced HF have been heavily used for >40 years. The use of dog models has diminished over time in part due to its extensive collateral circulation in dogs, which differs dramatically from that of humans and other large animals (e.g., pigs) (53). We referenced several historical studies that provide a foundation for functional and structural adaptations to the myocardium and coronary vasculature in aortic banded dogs (54–58).

**SYSTEMIC HYPERTENSION MODELS.** Historical studies led to the development of canine models of systemic hypertension by clamping of a renal artery (59) and wrapping 1 or 2 kidneys in cellophane (60). These models of systemic pressure overload have been used to study several aspects of HFpEF in both young and importantly, old dogs, given the significant aging component associated with this type of HF (45). Use of the “Page model” of bilateral renal wrapping for 6 to 12 weeks (both with and without deoxycorticosterone acetate) induced hypertension with systolic blood pressure reaching as high as 250 mm Hg, concentric LV remodeling, preserved EF, diastolic dysfunction, and fibrosis (61–64). Classic signs of experimental HF were mostly absent, and
comorbidities often associated with HFpEF were not incorporated into the model.

Two more recent swine models induced systemic pressure overload via hypertension using a combination of factors layered with comorbidities relevant to HFpEF. Sorop et al. (65) combined systemic hypertension (renal artery embolization), diabetes (streptozotocin), and hypercholesterolemia (high-fat diet) in 2- to 3-month-old Yorkshire-Landrace swine for 6 months. Renal embolization was achieved by injection of 75 mg of 38- to 42-μm polyethylene microspheres into the right kidney and into one-third of the left kidney. These animals, also listed by the National Heart, Lung, and Blood Institute HFpEF working group as a multihit model of HFpEF (44), demonstrated hemodynamic indicators of experimental HF, including increased left atrial pressure per a given cardiac index at rest and during exercise. Ejection fraction was more in the mid-range in this model (45%) and observed in parallel with diastolic dysfunction (increased end-diastolic pressure-volume relationship, titin isoform shift), increased LV collagen deposition, and coronary microvascular dysfunction. Obesity and concentric LV hypertrophy were absent, despite evidence of systemic increases in blood pressure, inflammation (plasma tumor necrosis factor–α), and metabolic derangement (type 1 diabetes, dyslipidemia).

The second of these studies included the combination of deoxycorticosterone acetate with Western diet for 12 weeks in Landrace swine (66). Hemodynamic markers of experimental HF were evident as a decrease in cardiac output and increase in LV EDP in response to pacing or dobutamine under anesthesia. Obesity, dyslipidemia, and increased systolic blood pressure were seen in combination with concentric LV hypertrophy, normal EF, diastolic dysfunction (titin isoform shift and altered phosphorylation), and LV nitric oxide synthase uncoupling. A separate magnetic resonance study in this same model also demonstrated impaired myocardial perfusion reserve, longitudinal strain, and torsion in response to dobutamine stress (67).

**HF INDUCED BY MYOCARDIAL INFARCTION**

Cardiac cell death associated with aberrant heart dysfunction is the main characteristic of a myocardial infarction (MI), which can ultimately lead to HF (68,69). This catastrophic event occurs due to interruption of blood flow to a discrete area of the myocardium that results from partial or complete occlusion of 1 or multiple coronary arteries. Unbalanced myocardial blood supply and demand can be spontaneously precipitated by coronary artery disease (e.g., atherosclerosis or thrombosis) or can occur during periprocedural revascularization surgery performed to revert spontaneous ischemia (e.g., percutaneous coronary intervention [PCI] or coronary artery bypass grafting) (68,69). The correct identification of different types of MI in response to ischemia is critical for optimizing patient treatment and is an important consideration for translational studies attempting to model acute MI and the subsequent development of HF. In this regard, the Universal Definition of MI was recently updated based on critical clinical projections driven by MI, including biomarkers (e.g., cardiac troponin levels), pathological features (e.g., edema, reduced glycogen content, and mitochondrial abnormalities), electrocardiography (e.g., new ST-segment elevations), and imaging by echocardiography, radionuclide imaging, or resonance magnetic imaging (e.g., myocardial free wall rupture and mitral regurgitation) (70).

Cardiac structural, functional, and metabolic characterization after MI reveal disruption of the contractile apparatus, mitochondrial impairment, endothelial dysfunction, and increased cell death (71,72). Coronary revascularization procedures such as PCI or coronary artery bypass grafting can improve survival rate post-MI and quality of life, but often result in the development of decompensated HF (i.e., HFrEF) (73,74). Consistent with this progression of HF, animal models of MI-induced HF are characterized by an initial ischemic event followed by a decrease in cardiac output and reduced EF, ventricular dilation associated with normal or reduced wall thickness (i.e., eccentric hypertrophy), areas of focal fibrosis in the ischemic area, activation of neurohormonal systems, and decreased cardiac reserve (17).

Experimental models of MI-induced HF include ischemia/reperfusion (I/R), non-reversible coronary occlusion induced by coronary ligation or ameroid constrictors, and coronary microembolization. Each technique incorporates clinical features that encompass central and peripheral modifications observed in patients with HFrEF caused by MI. An I/R approach acutely occludes coronary blood flow to the myocardium followed by reintroduction of blood flow to the ischemic area (75,76). Molecular mechanisms driving reperfusion injury are sudden arrhythmias, myocardial stunning caused by calcium overload and oxidative stress, as well as microvascular and endothelial dysfunction (74,77). This method has been most often used in the left anterior descending coronary artery (LAD) or left circumflex coronary artery (LCx) by
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reversible ligation or inflatable angioplasty balloon. Although historically dogs were used in I/R studies, an extensive coronary collateral circulation present in the canine heart has significantly decreased the use of this model. In pigs and sheep, strengths of I/R approaches include the ability to create infarcts of a predictable size and location by taking advantage of similarities more reminiscent of human coronary arteries, including gross anatomical structure and an absence of existing collateral vessels. Weaknesses of swine and ovine I/R models include significant acute susceptibility to arrhythmia and difficulty imaging the heart using ultrasound techniques due to ruminant-dependent differences in gastrointestinal anatomy.

Non-reversible coronary occlusion is performed by suture ligation or ameroid constrictor placement without reperfusion. The coronary ligation by suture is an immediate approach to develop acute MI, whereas ameroid constrictors can mimic MI resulting from coronary stenosis due to progressive atherosclerotic plaque formation. Limitations to this technique include permanent occlusion of vascular flow to the myocardium, which is rarely seen clinically because routine treatment includes reperfusion of the ischemic myocardium via PCI or coronary artery bypass grafting.

Finally, coronary microembolizations are sequential injections of microspheres that can be performed acutely and/or over time (78,79). Accumulation of atherosclerotic plaque debris in the coronary microcirculation increases the incidence of microembolization, varying from 20% to 79% (80), which can also result from PCI (81). Disruption of coronary atherosclerotic plaques by rupture, erosion, or calcific nodules can release harmful substances and potentially aggregate in the distal coronary microcirculation, which causes vasoconstriction, inflammation, and potential microinfarcts (79,82). Currently, clinical evidence of atherosclerosis driving reduction of myocardial blood flow and incidence of MI in the absence of significant coronary occlusion is classified as MI in the absence of coronary artery disease (83–85). Atherosclerotic plaque disruption and subsequent coronary microembolization can impair myocardium contractility and increase inflammation predominantly mediated by tumor necrosis factor–α, with sustained embolization resulting in repetitive events of thrombogenesis leading to MI. Although this technique can model the chronic effects of gradually increasing ischemia to the myocardium over time, consistency and reproducibility of infarcts can be difficult because of multiple embolization surgical procedures and a limited ability to control the extent of occlusion throughout the coronary vascular tree. Furthermore, this model results in multiple infarct and remodeling sites in the myocardium (in contrast to an individual area with a focused distinct injury) that can introduce variability and inconsistency to the assessment process. The following sections examine these models of MI-induced HF in large animals and outline physiological and molecular phenotypes relevant to each technique.

I/R MODELS. Occlusion times of coronary arteries from 30 to 180 min in duration have been shown to cause an ischemic insult significant enough to induce myocardium cell death (70,71,75). Given extensive coronary anastomosis has resulted in the decreased use of dogs to study MI and subsequent HF (53,76), a recent study investigated the effects of LV mechanical unloading after reversible LAD coronary ligation plus ligation of branches originating from the LCx coronary artery that potentially feed the LAD coronary area (86). With the stated goal of preventing potential influence of collateral circulation, coronary arteries were ligated for 180 min and then reperfused. Four weeks post-MI, this method produced an infarcted area of approximately 16% and a LVEF of approximately 40% in parallel with increased LV EDP, LV end-systolic volume, and N-terminal pro–B-type natriuretic peptide suggestive of HFrEF. This I/R approach provides a new alternative method that may help account for coronary collateralization in dog models of MI-induced HF.

In Yorkshire swine, differences in MI-induced HF following occlusion of the proximal LAD or LCx was examined using an I/R protocol produced by inflation of an intracoronary angioplasty balloon for 120 min (87). After 3 months, occlusion of the LAD produced an infarct size of approximately 14% in contrast to approximately 10% after occlusion of the LCx coronary artery. Increases in LV weight and impairments to LV mechanics (torsion and radial and/or circumferential strain, assessed via 2-dimensional speckle tracking echocardiography) was the same between groups, with a greater decrease in LVEF and increased end-systolic and end-diastolic volumes observed in the proximal LAD occluded group. Overall, the I/R protocol that used proximal occlusion of the LAD resulted in more severe disease, which suggested it may be a better preclinical model of MI-induced HF. Other studies in swine showed impaired calcium handling (decreased calcium transient amplitude and increased diastolic calcium levels) 14 weeks post-1/R using occlusion of the proximal LCx coronary artery for 2 h, which resulted in an EF of 39% (88).
Ovine models of I/R were also used to examine MI-induced HF. Charles et al. (89) examined I/R in Coopworth ewes using 90 min of occlusion by intra-coronary balloon angioplasty placed between the first and second diagonals of the LAD coronary artery (89). Acute increases in natriuretic peptide level and cardiac troponin T (peak at 7 h post-MI) were observed alongside reduced EF (38%) 7 days post-MI, although LV dilation was not observed. In Dorset hybrid sheep, infarction size was dependent on time of ischemia (range 45 min to 6 h) with damage susceptibility significantly influenced by regional myocardial location (90). Six hours of ischemia progressively increased LV volume and decreased EF (27%) in Dorset hybrid sheep 12 weeks post-MI (91). Recent work demonstrated a decrease in EF (38%) 2 weeks post-MI following 120 min of occlusion in the LCx coronary artery using balloon occlusion (92).

NON-REVERSIBLE CORONARY OCCLUSION MODELS. van der Velden et al. (93) studied MI caused by non-reversible LCx artery ligation in 2- to 3-month-old Yorkshire-Landrace pigs. Three weeks post-MI, permanent ligation of the LCx artery increased heart weight, end-diastolic and end-systolic areas, and significantly decreased EF (35%). The sarcoplasmic/endoplasmic reticulum calcium-ATPase 2a (SERCA2a) protein level was decreased, and skinned cardiomyocytes demonstrated decreased maximal force generation and increased calcium sensitivity believed to be mediated by altered PKA phosphorylation of troponin I. A separate group of studies in Yorkshire swine (45 days old) demonstrated permanent ligation of the LCx or LAD coronary artery decreased EF (35%). The bioenergetic reservoir measured by concentration of high-energy phosphate levels (phosphocreatine/adenosine triphosphate ratio) 4 to 8 weeks post-MI (94,95).

Ameroid constrictors were also used with and without comorbidities such as obesity and type 2 diabetes in swine to induce MI and HF. Early studies placed 2.0- to 2.5-mm ameroid constrictors around the LCx coronary artery, with gradual occlusion of blood flow resulting in a highly variable infarct size (5% to 37% of the LV) (96,97). Recently, an ameroid constrictor was placed on the LAD coronary artery for 4 weeks in obese Ossabaw swine, which resulted in infarct sizes of approximately 15% (98). Other studies that examined obese Ossabaw swine placed an ameroid constrictor around the LCx for 7 weeks, which resulted in a model of chronic ischemia as opposed to MI-induced HF because no infarcts were observed (99,100).

Ovine models of ischemic heart disease induced by ameroid constrictor were also used. Chekanov et al. (101) analyzed the effects a 3.5-mm ameroid constrictor placement in the LCx coronary artery. After 4 weeks, EF decreased to 49% and was associated with an increase in LV end-diastolic and end-systolic volumes. A separate set of studies in Coopworth ewes showed significant decreases in EF% (20% to 25%) 1 to 4 weeks post-surgery after occluding the LAD coronary artery using a thrombogenic coil in parallel with increased plasma circulating natriuretic peptides, cardiac troponin T, and creatine kinase (89,102).

CORONARY MICROEMBOLIZATION. Attempting to replicate acute MI and subsequent HF in a dog model of coronary microembolization, Franciosa et al. (103) acutely injected 100-μl glass microspheres (approximately 475 μm in diameter) into the LCx coronary artery of mongrel dogs and evaluated the animals at 1, 3, and 10 months. Ten months post-surgery, MI (23% average scar size) was associated with a significant decrease in cardiac output (103). Later studies used polystyrene latex microspheres (77 to 102 μm in diameter) sequentially injected into the LAD and LCx coronary arteries over 1 to 3 weeks (3 to 9 total coronary embolization procedures/animal) (104). Three months post-surgery, cardiac output and EF (21%) were decreased and associated with a transmural MI distributed throughout the LV, septum, and RV. Signs of HF included LV dilation (increased end-diastolic volume), increased pulmonary artery wedge pressure, and increased plasma atrial natriuretic peptide and/or norepinephrine.

A cardiac magnetic resonance study infused microbeads 100 to 300 μm in diameter into the LAD coronary artery of farm pigs (34 kg), which resulted in decreased EF (36%) 1 week after microembolization (105). In 4- to 5-month-old Yucatan mini-swine, Hanes et al. (106) acutely injected 2 ml of 90 μm polystyrene microspheres into the LAD coronary artery. This model was used in a follow-up study, which demonstrated decreased EF (45%) and LV dilation (increased end-diastolic volume) in addition to significant electrophysiological remodeling of numerous myocardial ion currents (106). Recent studies also used microspheres to induce acute MI by injecting 1 ml of polyvinyl-alcohol microspheres (45 to 150 μm in diameter) every 3 to 10 min for 45 min total in pigs (107).

In adult Merino Wether sheep (51 kg), an average of 5 embolization procedures was administered every 2 weeks using 90-μm polystyrene microspheres in both the LAD and LCx coronary arteries (108,109).
Continuous testing and evaluation of safety and/or efficacy during the preclinical phase is a proposed pathway to improving clinical success. (Intra-aortic balloon pump [IABP], Impella, and TandemHeart pictures from were originally published in Atkinson et al. [170]). FDA — Food and Drug Administration.
Several indicators of HF were observed 26 weeks post-procedure, including decreased EF (27%), increased pulmonary capillary wedge pressure, and LV dilation and wall thinning (increased end-diastolic volume and decreased LV wall thickness, respectively). Monreal et al. (110) injected 0.5 ml of 90-μm fluorescence polystyrene microspheres into the LCx coronary artery in Dorsett cross sheep (44 kg). This approach successfully reduced EF (25%), caused LV dilation (increased end-diastolic volume), and increased mean pulmonary artery pressure 1 week post-surgery. Scar size was significantly increased in these animals compared with 90 min of I/R alone.

**HF INDUCED BY ARRHYTHMIA**

The pathological interaction between arrhythmia and HF is well established, increasing both the risk of developing HF and morbidity and/or mortality in established HF cases (112). Recently, arrhythmia-induced cardiomyopathy was proposed as a more inclusive way to examine the diverse impact of electrophysiological pathology to the overall HF syndrome (113). Sudden cardiac death is significant cause of mortality in HF, regardless of EF, and the role of tachycardia to developing HF has long been appreciated. In particular, supraventricular arrhythmias such as atrial fibrillation (AF) can increase HF risk 3-fold (114-116). Similar to the human syndrome, the development of HF in animal models of arrhythmia-induced cardiomyopathy include bi-ventricular dilation and decreased wall thickness, followed by a steady deterioration of cardiac output and EF over time, activation of neurohormonal systems, and significant impairment of cellular calcium homeostasis. Experimental models of arrhythmia-induced HF are characterized by periods of chronic rapid pacing denoted primarily by anatomical location of the pacemaker. For tachycardia models, the pacemaker is implanted in the RV or LV, whereas animal models of AF often stimulate pathological pacing of the myocardium from an atrial location. Interestingly, arrhythmia-induced models of HF models include almost complete recovery of myocardial function and structure upon termination of the pacing stimulus. Important considerations for these techniques include: 1) changes to myocardial structure and function, which can vary significantly within the same heart based on proximity to the pacemaker; and 2) development of HF directly related to pacing rate and duration. Furthermore, atrial pacing has demonstrated an inability to sustain chronic AF for longer than 2 to 8 weeks and often requires parallel dosing of traditional cardiac therapeutics, including β-blockers and/or cardiac glycosides. Because of the historical use of these models, we briefly highlight these approaches in the following sections.

**PACING-INDUCED TACHYCARDIA.** Originally reported in 1962 (117), a significant number of canine (118,119), swine (120,121), and ovine (122,123) models of pacing-induced HF have been developed and used in both acute and chronic experimental settings (references provided for historical context). Cardiac pacing using heart rates across a spectrum of 120 to 260 beats/min have been routinely shown to induce symptoms comparable to HFrEF, including decreased EF, ventricular dilation and decreased ventricular wall thickness, pulmonary involvement, increased plasma expression of biomarkers (e.g., natriuretic peptides), and neurohumoral activation. Recently, Möllmann et al. (124) desynchronized heart beats in swine (10 to 12 weeks old; 34 kg) using different pacing locations in the RV (110 beat/min for each lead, effective heart rate of 220 beats/min). Desynchronization caused more severe HF compared with single-lead pacing, which was characterized by significantly impairing LV systolic function (decreased cardiac and/or stroke volume index and fractional shortening), increased pulmonary capillary wedge pressure and LV end-diastolic dimension, cardiac hypertrophy, and pathological ECM remodeling.

**ATRIAL FIBRILLATION.** Although several large animal models of AF have been developed, studies in which this common arrhythmia results in HF are limited. Dosdall et al. (125) examined the impact of chronic rapid atrial pacing in mixed breed hounds, Boer and mixed breed goats, and Yorkshire swine in a study designed to determine the appropriate animal model to optimize long-term development of AF and subsequent HF. A pacemaker implanted in the right atria was programmed to stimulate at 50 Hz for 1 s followed by 1 s of no stimulation at 2 to 3 times the diastolic pacing threshold. Every 1 to 2 weeks, the pacemaker was deactivated to determine whether
sustained AF had developed. If AF was sustained, the pacemaker was reprogrammed to stimulate AF only if the animal returned to normal sinus rhythm. Six months post-intervention, dogs were the only animals to develop signs of HF evident as a decrease in EF to 30% 3 weeks or 3 months after the initiation of pacing, respectively (126,127). In sheep, the combination of atrial and RV pacing impaired LV contractility and relaxation during exercise (±dP/dt_{max}) and increased biomarkers of HF (brain natriuretic peptide, endothelin) 21 days after introduction of the arrhythmic stimulus (128).

### The Translational Role of Large Animal Models of HF: A Critical Part of Clinical Success?

Pre-clinical large animal models of cardiovascular disease are an essential, but arguably underserved translational bridge to the development and testing of new therapies and devices before clinical trials (129). Clinical attrition rates for research and development range from 80% to 97%, with clinical segments of the overall studies accounting for 73% of the total cost of bringing the therapeutic to market (130). This high rate of failure occurs despite preclinical attrition rates of only 35%, which suggests high levels of success in animal research may be “fool’s gold.” Several factors have been proposed regarding the failure of animal research to translate clinically, including overoptimistic conclusions inferred from methodologically flawed animal studies, animal models that do not adequately reflect human disease, and neutral or negative outcomes in animal studies that are more likely to remain unpublished (131,132). Together, these factors contribute to a likelihood of an approval rate of approximately 7% for cardiovascular drugs as assessed from phase I clinical trials to official authorization of new drug and/or biologic license application (133). Currently, >60% of the time and money required for the successful approval of a new device or therapeutic is spent during human clinical trials (134). Given the high rate of failure during this phase, studies have suggested that an increase in the amount of time and money spent on candidate selection, safety, and efficacy in the preclinical phase could improve human translation both methodologically and economically (134). Recent viewpoints have proposed follow-up times of ≥1 year in large animal studies to better assess endpoint success pre-clinically before moving to phase I (129). Large animal models of pathophysiology are also necessary to improve translation practically by: 1) facilitating testing of clinical delivery, imaging, and support devices; 2) providing valuable toxicology and

| TABLE 2 Summary of Clinical Trials for Mechanical Circulatory Support Devices and Associated Preclinical Large Animal Studies |
|---------------------------------------------------------------|
| **Mechanical Circulatory Support Device/Clinical Trial Name** | **Clinical Trial Identifier** | **Clinical Trial status** | **Patients** | **Large Animal Studies** |
|---------------------------------------------------------------|
| Impella System* (2.5; CP; LD; LP 2.5) DTU | NCT03300270 | Completed | 50 Pig (152,156-161); sheep (162,163) |
| REVERSE | NCT03431467 | Recruiting | 96 |
| PERMITT | NCT01294267 | Completed | 20 |
| RECOVER I | NCT00596726 | Completed | 17 |
| PROTECT I | NCT00534859 | Completed | 28 |
| CARDSUP | NCT04117230 | Recruiting | 1500 |
| Protect Kidney Trial | NCT04321148 | Recruiting | 224 |
| ISAR-SHOCK | NCT00417378 | Completed | 26 |
| Protect PCI Study | NCT02831881 | Recruiting | 369 |
| TandemHeart† | NCT02326402 | Recruiting | 200 Pig (151-155) |
| ANCHOR | NCT04184635 | Active/Not yet recruiting | 400 |
| CentriMag Circulatory† | NCT00819793 | Completed | 32 Sheep (164-166); pig (167) |
| Failure-to-Wean | NCT00819793 | Completed | 25 |
| CMagRVAS | NCT01568424 | Completed | 25 |

*Food and Drug Administration (FDA) approval: P140003 and 510k-K063723. FDA approval: 510(k)-K10493. FDA approval: recalled. ANCHOR = Assessment of ECMO in Acute Myocardial Infarction Cardiogenic Shock; CARDSUP = Swiss Circulatory Support Registry; CMagRVAS = CentriMag RVAS U.S. Post-approval Study Protocol; DTU = Door To Unloading With IMPELLA CP System in Acute Myocardial Infarction - Safety and Feasibility Study; Failure-to-Wean = CentriMag Ventricular Assist System in Treating Failure-to-Wean From Cardiopulmonary Bypass; ISAR-SHOCK = Efficacy Study of LV Assist Device to Treat Patients With Cardiogenic Shock; PERMITT = Percutaneous Hemodynamic Support With Impella 2.5 During Scar-related Ventricular Tachycardia Ablation; PROTECT I = A Prospective Feasibility Trial Investigating the Use of IMPELLA RECOVER LP 2.5 System in Patients Undergoing High Risk PCI; RECOVER I = RECOVER I Impella RECOVER LP/LD 5.0 Support System Safety and Feasibility Study; REVERSE = Impella CP With VA ECMO For Cardiogenic Shock; THEME = TandemHeart Experiences and Methods.
biodistribution information; and 3) providing relevant physiological inputs that can guide computational and omics-based assessment of clinical risk useful for precision medicine. Supporting data from large animal models is often a critical aspect of the investigational New Drug or Device Exemption process that leads to FDA approval. Thus, the following section highlights recent clinical trials that have used preclinical studies as part of the development process for new HF therapeutic support devices (Figure 2).

The FDA evaluates, regulates, and approves various medical products, including mechanical circulatory support devices for patients with HF, such as intra-aortic balloon pumps (IABPs), axial flow pumps, and left atrial-to-femoral arterial ventricular assist devices. These devices mechanically assist the myocardium, providing short-term systemic hemodynamic support and minimizing myocardial workload during ischemic events complicated by cardiogenic shock or during high-risk PCI procedures (135-137). IABPs were previously considered class I treatment for acute MI complicated by cardiogenic shock (138-140) but are currently recommended as class II treatment (141,142). Although widely used in patients with HF, IABPs have hemodynamic and surgical limitations (143-145). As a result, new mechanical circulatory support devices have been developed for clinical use. These devices support cardiac function by collecting blood from: 1) the LV and delivering it into the ascending aorta (135,146,147); or 2) the left atrium and delivering the blood to the femoral artery by centrifugal bypass (135,148-150). Two of these devices are the FDA-approved TandemHeart (left atrial-to-femoral arterial ventricular assist device) (LivaNova PLC, London, United Kingdom) and Impella (Axial flow pump) LV support systems (Abiomed Inc., Danvers, Massachusetts). TandemHeart supports systemic hemodynamics by pumping blood up to 4 l/min, whereas several Impella models (Impella CP, Impella 2.5, Impella 5.0/LD, and Impella RP) provide flow ranging from 2.5 to 5.0 l/min. The efficacy and safety of these systems has been tested in several large animal models of HF, which are reported adjacent to their associated clinical trials in Table 2.

The impact of TandemHeart on hemodynamics and cardiac morphology has been investigated in porcine models of acute MI or ventricular arrhythmia (151–155). For example, TandemHeart was implanted during LCx occlusion (30 min) and effectively unloaded the LV while maintaining systemic pressure, which was evident via decreased stroke volume, end-diastolic volume, and EDP (151). Impella devices have also been investigated in swine (152,156-161) and ovine (162,163) models of adult and pediatric HF. Using their recently described combination I/R–coronary microembolization protocol in Yorkshire swine, Watanabe et al. (159) examined mechanical LV unloading 2 weeks post-MI using the Impella CP. After 2 h of circulatory support, Impella decreased LV end-diastolic volume and EDP, maintained peripheral vascular pressure, and increased coronary vascular perfusion in the infarct area. An interesting comparison between Impella and TandemHeart was assessed in Yorkshire swine (76 kg) subjected to acute MI induced by occlusion of the LCx for 2 h followed by 30 min of reperfusion (152). At comparable flow rates, TandemHeart decreased LV preload (end-diastolic volume), stroke volume, and contractility (dP/dt max, stroke work, pre-load recruitable stroke work) to a greater extent than the Impella.

Although the preceding studies reflect successful translational interactions and outcomes between large animal and human studies, the process is not infallible. One example is the CentriMag Circulatory ventricular assist device (Abbott Laboratories, Abbott Park, Illinois), which was recalled due to a calibrating system error linked to electromagnetic interference, which caused the device to stop (https://www.fda.gov/medical-devices/medical-device-recalls/abbott-recalls-centrimag-circulatory-support-system-motor-due-pump-and-motor-issues). Difficulties with the device occurred after FDA approval, despite several preclinical studies in both sheep (164–166) and pigs (167), 3 registered clinical trials (Table 2) (CentriMag Ventricular Assist System in Treating Failure-to-Wean From Cardiopulmonary Bypass; NCT00819793 [completed]; CentriMag Ventricular Assist System in Treating Failure-to-Wean From Cardiopulmonary Bypass for Pediatric Patients; NCT01171950 [withdrawn]; and CentriMag RVAS U.S. Post-approval Study Protocol [CMagRVAS]; NCT01568424 [completed]), and a multicenter study that showed short-term support with low incidence of device-related complications and no device failure in cardiogenic shock patients 30 days after CentriMag implantation (168). These findings are not meant to
undermine the importance of integrating preclinical and clinical studies for assessing safety and feasibility during development, but rather to highlight that work still remains regarding optimization of current systems that facilitate testing and/or approval of new therapies and devices.

CONCLUSIONS

Preclinical large animal models play a critical and expanding role in translating basic science findings to the development and clinical approval of novel cardiovascular therapeutics. As recently examined in mice (169), researchers are similarly encouraged to consider the strengths and weaknesses of large animal models, including specific breeds, comorbidities, disease modifiers, and overall study goals such as acute or chronic outcomes. Space, cost, and competencies should also be taken into consideration given differences in U.S. Department of Agriculture requirements for large animal housing and surgical expertise can vary greatly between large animal species. Increasing the use of large animal models of HF holds significant potential to identify novel mechanisms underlying the HF condition, to provide valuable information regarding the safety and efficacy of new therapies, and to improve physiological and economical translation of animal research to the successful treatment of human HF.

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**KEY WORDS** heart failure, HFrEF, HFrEF, large animal model, preclinical
Autosis
A New Target to Prevent Cell Death

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HIGHLIGHTS

- Autosis is an autophagy-dependent, nonapoptotic, and non-necrotic form of cell death that is characterized by unique morphological and biochemical features, including the presence of ballooning of perinuclear space (PNS) and sensitivity to cardiac glycosides, respectively.
- Autotic cell death may be initiated by excessive accumulation of autophagosomes rather than lysosomal degradation.
- Autosis is stimulated during the late phase of reperfusion after a period of ischemia in the heart when up-regulation of rubicon in the presence of continuous autophagosome production induces massive accumulation of autophagosomes.
- Suppression of autosis, which may reduce death of cardiomyocytes during the late phase of reperfusion, in combination with inhibition of apoptosis and necrosis targeting the early phase of injury, may enhance the effectiveness of treatment for I/R injury in the heart.

SUMMARY

Excessive autophagy induces a defined form of cell death called autosis, which is characterized by unique morphological features, including ballooning of perinuclear space and biochemical features, including sensitivity to cardiac glycosides. Autosis is observed during the late phase of reperfusion after a period of ischemia and contributes to myocardial injury. This review discusses unique features of autosis, the involvement of autosis in myocardial injury, and the molecular mechanism of autosis. Because autosis promotes myocardial injury under some conditions, a better understanding of autosis may lead to development of novel interventions to protect the heart against myocardial stress. (J Am Coll Cardiol Basic Trans Science 2020;5:857–69) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Macroautophagy (hereafter autophagy) is an essential process by which cells degrade intracellular organelles and cytosolic materials, characterized by the presence of double-membrane structures called autophagosomes that fuse with lysosomes. Traditionally, autophagy is believed to be a protective mechanism because it eliminates misfolded or dysfunctional proteins or organelles and supplies energy by recycling the amino acids and fatty acids obtained through degradation (1). However, emerging evidence suggests that the genetic machinery of autophagy is also essential for cell death under some conditions (2). Cell death mediated by autophagy, or autophagic cell death,
was originally classified as a type II programmed cell death, defined by massive cytosolic vacuolization in dying cells. Autophagic cell death has been reported in cardiomyocytes during ischemia-reperfusion (I/R), pressure overload, and doxorubicin-induced cardiomyopathy (3-5). However, because of technical limitations that make it difficult to distinguish autophagic cell death from other forms of death accompanied by autophagy, the causative role of autophagy in cardiomyocyte cell death is largely unknown. Recently, a unique form of cell death induced by activation of autophagy was identified and termed autosis (6). Autosis is distinct from other forms of cell death, including necrosis and apoptosis, and is characterized by the presence of unique morphological and biochemical features. Increasing lines of evidence suggest that cell death through autosis occurs in various cell types and organs in response to some types of stress. We have also shown recently that cardiomyocytes die by autosis during reperfusion after a short period of ischemia in the heart (7,23). Here, we review the current understanding of autophagic cell death, with a focus on autosis, and we discuss the involvement of cardiomyocyte autosis in myocardial injury and the underlying molecular mechanisms.

AUTOPHAGY AND AUTOPHAGIC CELL DEATH IN THE HEART

To explain how autophagy can kill cells under some conditions, we will first describe the physiological function of autophagy and its molecular machinery. Autophagy is an essential catabolic process that is highly conserved in eukaryotes. The autophagy pathway primarily consists of evolutionarily conserved autophagy-related (ATG) proteins. These ATG proteins generate double-membrane vesicles, termed autophagosomes, which engulf cellular materials, generally in a nonselective manner but also in a selective manner under some conditions. The autophagy process is initiated by activation of the Unc-51-like kinase 1 (Atg1) complex, which is regulated by mammalian target of rapamycin complex 1 and adenosine monophosphate-activated protein kinase (8). The Unc-51-like kinase 1/Atg1 complex activates the class III phosphatidylinositol 3 kinase (PI3K) complex, consisting of PI3K, beclin 1, vacuolar protein sorting 15 (VPS15), and/or additional partners, to initiate autophagosome nucleation. As a scaffold protein, beclin 1 recruits several proteins, such as Atg14L, autophagy and beclin regulator 1 (AMBRA1), ultraviolet resistance-associated gene (UVRAG), and rubicon, to the nucleation site to regulate the lipid kinase activity of the PI3K complex (9). Although most beclin 1 interactors enhance beclin 1 activity, rubicon negatively regulates both the autophagic and endocytic pathways by interacting with beclin 1 or Rab7, a member of the RAS oncogene family (10). Unlike in yeast, there is no pre-autophagosomal structure in mammalian cells. Instead, the endoplasmic reticulum (ER) exit sites, mitochondria, ER-mitochondria contact sites, the ER-Golgi intermediate compartment, the Golgi apparatus, and the plasma membrane have all been reported as sources of isolation membranes in mammalian cells (11). Whether dysregulated autophagosome formation leads to depletion of the aforementioned structures and contributes to malfunction of intracellular organelles is an open question. In subsequent steps, elongation and completion of autophagosomes are regulated by the ubiquitin-like conjugation system. First, the E1-like enzyme Atg7 binds to the ubiquitin-like protein Atg12, which promotes conjugation of Atg12 to Atg5 in the presence of an E2-like enzyme, Atg10, finally forming the Atg5-Atg12/Atg16 multimeric complex. The Atg5-Atg12/Atg16 complex localizes at expanding phagophores and acts as an E3-like ligase to conjugate another ubiquitin-like molecule, Atg8 (light chain 3-I), to phosphatidylethanolamine to form LC3 (MAP1LC3; a light chain of the microtubule-associated protein 1, ortholog of yeast Atg8), which is essential for targeting autophagosomes to lysosomes (12). Finally, autophagosomes fuse with lysosomes to degrade the sequestered materials. On completion of autophagosome formation, syntaxin 17, localized on the outer autophagosomal membrane, interacts with synaptosomal-associated protein receptor vesicle-associated membrane protein 8 to enable fusion with the lysosomes (13) (Figure 1). Within the lysosomal compartment, membrane components and proteins are degraded by phospholipases and proteolytic enzymes, respectively, and degraded phospholipids and amino acids are recycled (14). Because autophagy is executed through the coordinated actions of: 1) autophagosome formation; 2) fusion between autophagosomes and lysosomes; and 3) degradation of autophagosomes and its cargo in lysosomes, dysregulation of any step could potentially induce malfunction of autophagic degradation and disturb cellular homeostasis, thereby leading to cell
death. For example, blocking autophagy at the level of lysosomal degradation could lead to accumulation of undigested autophagosomes and depletion of membrane sources that would otherwise be recycled to form new autophagosomes.

Autophagic cell death was originally identified by a massive cytoplasmic vacuolization with autophagy activation (2). It is important to note that the presence of autophagic vacuoles in dying cells does not necessarily mean that autophagy promotes cell death. Because autophagy is often activated in response to stress as an adaptive mechanism, the presence of autophagy in dying cells could indicate that the last resort to prevent cell death may have failed. Unfortunately, because most of the currently available inhibitors are not 100% specific for autophagy, chemical inactivation of autophagy may not provide definitive evidence of autophagic cell death. Similarly, many
molecules involved in autophagy, such as Atg, have autophagy-independent functions (15), which makes the use of genetically altered animal models for loss-of-function studies of autophagy challenging as well. For these reasons, autophagy-dependent cell death has been defined by the Nomenclature Committee on Cell Death as a cell death that is suppressed by at least 2-independent interventions to inhibit the autophagy pathway (16).

With these technical challenges in mind, under what conditions is autophagy involved in cell death? In many examples, autophagy is activated strongly, and molecules involved in autophagosome formation, including beclin 1, are up-regulated when autophagy-dependent cell death takes place. For example, these conditions are observed in the heart in response to some pathologically relevant stresses, including I/R, acute pressure overload, and doxorubicin-induced cardiotoxicity. It is noteworthy that several interventions to suppress the autophagic machinery, including down-regulation of beclin 1 and chemical inhibitors of autophagy such as 3-methyladenine, an inhibitor of class III PI3K activity and autophagosome formation, significantly suppress death of cardiomyocytes and myocardial injury. If we include other types of cell death that are suppressed when autophagy is inhibited, autophagy-dependent cell death may also include the following forms of cell death. First, some forms of autophagy selectively degrade proteins essential for cell survival. For example, in flies, autophagy selectively degrades Drosophila BIR repeat containing ubiquitin-conjugating enzyme (dBruce) dBruce, a caspase inhibitor, thereby activating caspase-dependent cell death, namely apoptosis (17). Autophagy may also degrade catalase, an antioxidant, which in turn disrupts the intracellular redox balance and induces cell death in mouse fibroblast cell lines (18). Second, activation of nonselective autophagy in a mouse model of diabetes reciprocally suppresses mitophagy, thereby inducing mitochondrial dysfunction and death in cardiomyocytes. Inhibition of autophagy by down-regulation of Becn1 or Atg16 improves diabetic cardiomyopathy by activating mitophagy through an unknown mechanism (19). Although autophagy is involved in these 2 types of cell death, the cell death itself is mediated through other forms of cell death, including apoptosis and necrosis. It has thus been recommended to use the terms “autophagy-associated cell death” or “autophagy-mediated cell death” unless the death is prevented only by multiple autophagy-inhibiting interventions but not by inhibition of other forms of cell death, such as apoptosis or necrosis. Along these lines, it is preferable to use the term “autophagy-dependent cell death” when the aforementioned criteria have been clearly proven (20).

**NEW FORM OF AUTOPHAGIC CELL DEATH, AUTOSIS**

Although autophagy is associated with multiple forms of cell death as discussed, an important question remains as to whether autophagy-dependent cell death has any morphological or biochemical features that distinguish it from other forms of cell death. Recently, Liu et al. (6) characterized a new form of autophagy-dependent cell death, which is triggered by high doses of autophagy-inducing peptides, starvation, and permanent brain ischemia, conditions in which autophagy is strongly activated. This form of cell death, termed autosis, is characterized by the presence of unique morphological and biochemical features and can be rescued by inhibitors of autophagy, but not by inhibitors of apoptosis or necrosis. Thus, autosis clearly fulfills the criteria of autophagy-dependent cell death. Although autosis may not be the sole form of autophagy-dependent cell death, it is certainly the most well-defined form of cell death in this category.

The unique morphological features of autotic cell death are induced in a time-dependent manner (Figure 2). In phase 1a, dilated and fragmented ER and an increased number of autophagosomes, autolysosomes, and empty vacuoles are observed. In phase 1b, a swollen perinuclear space (PNS) containing cytoplasmic materials and electron-dense mitochondria can be observed by transmission electron microscopy. In phase 2, the last step of autosis, cytoplasmic organelles are drastically decreased and focal nuclear concavity and focal ballooning of the PNS are observed (21). Cells that have died by autosis are generally more firmly attached to the culture dishes than are those that do not undergo autosis, and the increased adherence appears to be another morphological feature of autosis in vitro. To date, the best method for defining autosis is electron microscopy analysis. Alternatively, immunofluorescence assays can also help distinguish autotic cells by detecting fragmented ER or mitochondria with nuclear concavity.

Although autosis can be inhibited by chemical inhibitors of autophagy, including 3-methyladenine, and down-regulation of the autophagic machinery, such as beclin 1 and Atg7, it cannot be inhibited by inhibitors of apoptosis, necrosis, or any other form of programmed cell death. It should be mentioned that suppression of lysosomes fails to inhibit autosis in either HeLa cells or cardiomyocytes. This suggests that autosis may be induced by excessive activation of autophagosome formation, but not by excessive
degradation. We will discuss this issue in the section on the underlying mechanism of autosis.

Another important feature of autosis is its sensitivity to cardiac glycosides. A compound library screen revealed that inhibitors of \( \text{Na}^+\text{,K}^+\text{-adenosine triphosphatase} \) (ATPase) effectively suppress autosis (6, 22). Consistent with this finding, cardiac glycoside, a chemical inhibitor of \( \text{Na}^+\text{,K}^+\text{-ATPase} \), dramatically reduces Transactivator of transcription (Tat)–beclin 1-induced autosis in vitro and the autosis observed in neonatal rats subjected to brain ischemia in vivo (6). Moreover, injection of ouabain into cardiac glycoside-sensitive mice significantly reduces myocardial I/R injury in adult mice (23). We also noted that protein expression of \( \text{Na}^+\text{,K}^+\text{-ATPase} \) was up-regulated in the heart in response to I/R, the time course of which coincides with that of autosis. One caveat here is that \( \text{Na}^+\text{,K}^+\text{-ATPase} \) in rodent hearts is less sensitive to cardiac glycosides than that in human hearts is. Thus, it is difficult to test whether cardiomyocyte death can be rescued by cardiac glycosides in the murine heart. Humanized \( \text{Na}^+\text{,K}^+\text{-ATPase} \) \( \alpha_1 \) subunit knock-in mice can be used to overcome this issue (23). Alternatively, the \( \alpha_1 \) subunit of \( \text{Na}^+\text{,K}^+\text{-ATPase} \) can be down-regulated with short hairpin ribonucleic acid without obvious effects on contractility.

The features of autosis compared with other forms of programmed cell death are summarized in Table 1. Currently, no other criteria are available to distinguish autotic cell death from other forms of cell death. We expect, however, that progress in the field...
**TABLE 1** Comparison of Biochemical and Morphological Features Among Autosis, Apoptosis, and Necrosis

|                  | Autosis                              | Apoptosis                           | Necrosis                      |
|------------------|--------------------------------------|-------------------------------------|-------------------------------|
| **Biochemical features** | Inhibited by inhibitors of autophagy but not by inhibitors of apoptosis or necrosis | Caspase activation and internucleosomal DNA fragmentation | Rapid, extensive thiol oxidation and high level of intracellular Ca<sup>2+</sup> |
| **Regulators**   | ATG genes, rubicin, Na<sup>+</sup>,K<sup>-</sup>-ATPase | Caspases, PARP | RIP1, RIP3 |
| **Morphological features** | Focal ballooning of PNS, focal concavity of the nuclear surface, mild chromatin condensation | Nuclear chromatin condensation, fragmentation of DNA, irregularity of nucleus | Karyolysis and caspase-independent DNA fragmentation, lysis of nucleus, dilation of nuclear membrane |
| **Nucleus**      | Focal plasma membrane rupture         | Intact, altered orientation of lipids | Disrupted                        |
| **Plasma membrane** | Minor changes                         | Reduced (shrinkage)                  | Increased (swelling)             |
| **Mitochondria** | Electron-dense mitochondria, abnormal internal structure, and swollen mitochondria | Release of cytochrome c, mitochondrial dysfunction | Failure of ATP production, collapse of mitochondrial membrane potential, and mPTP opening |
| **Cell size**    | Ballooning of PNS under EM, increased adherence of cultured cells to culture dishes | Apoptotic bodies, pseudopod retraction | Swelling of ER and mitochondria, lysosome, and plasma membrane rupture |

**ATG** = autophagy-related; **ATPase** = adenosine triphosphatase; **DNA** = deoxyribonucleic acid; **EM** = electron microscopy; **mPTP** = mitochondrial permeability transition pore; **PARP** = poly adenosine diphosphate ribose polymerase; **PNS** = perinuclear space; **RIP1** = receptor interacting serine/threonine kinase 1; **RIP3** = receptor interacting serine/threonine kinase 3.

may provide additional criteria to help identify autosis more easily and precisely.

**AUTOSIS-INDUCING CONDITIONS**

Although autosis was initially identified in HeLa cells when very high levels of autophagy were induced by Tat–beclin 1 in vitro (6), autosis has also been observed in vivo under various conditions (Central Illustration).

Hypoxia-ischemia and reperfusion appear to be common conditions in which strong activation of autophagy and autosis are observed in multiple organs. Cerebral hypoxia-ischemia in the brain of neonatal rats promotes autosis, and cardiac glycosides, such as nerifolin, digoxin, and digitoxigenin, rescue autotic cell death in vivo (6). Autosis is also induced in mouse kidneys subjected to I/R (22). More recently, autosis was also observed in the heart and the cardiomyocytes therein during the late phase of I/R. The fact that inhibition of autosis with cardiac glycosides reduces the size of infarct in all of these models suggests that autosis is widely involved in tissue injury in response to I/R (23).

Although human immunodeficiency virus (HIV) ultimately kills infected cells, HIV-infected macrophages escape cell death (24). Interestingly, autophagy-inducing peptides, including Tat–beclin 1 and Tat–viral FLICE inhibitory protein-sz, preferentially kill chronically HIV-infected human macrophages, whereas the cell death is inhibited by knockdown of ATG genes and Na<sup>+</sup>,K<sup>-</sup>-ATPase sz (25). A major reservoir of HIV latent infection is resting central memory CD4<sup>+</sup> T cells. Under antiretroviral treatment, HIV escapes clearance by staying inside CD4<sup>+</sup> T cells (26). However, HIV-infected CD4<sup>+</sup> T cells are also selectively killed by autophagy-inducing peptides through autosis (27). Thus, autosis selectively kills HIV-infected macrophages and CD4<sup>+</sup> T cells. This is intriguing in that HIV utilizes autophagy proteins for replication (28) but down-regulates autophagy to avoid proteolytic degradation in activated CD4<sup>+</sup> T cells (29). The molecular mechanisms through which HIV-infected macrophages and CD4<sup>+</sup> T cells selectively undergo autosis remain to be elucidated.

Activation of autophagy in response to hypoxia has dichotomous functions in cancer cells (30,31). Autophagy inhibits cancer cell death 4 h after hypoxia but promotes cell death 72 h after hypoxia (21). Autophagic cell death at late time points of hypoxia showed morphological features of autosis and was inhibited by cardiac glycosides in A549 cell lines. During the initial stage of hypoxia, epidermal growth factor receptor (EGFR) is activated and interacts with beclin 1, which activates autophagy at physiological levels and contributes to survival of cancer cells. When hypoxia persists, however, EGFR is deactivated by autophagic degradation of caveolin 1 and dissociates from the beclin 1 complex, which in turn stimulates autophagic flux above physiological levels, thereby triggering autosis. The detailed mechanism
through which protein-protein interaction between beclin 1 and EGFR regulates the level of autophagic flux and autosis is not well understood. Whether cancer cells become susceptible to autosis in response to prolonged hypoxia due to higher autophagic activity alone or whether additional modulatory mechanisms are needed to promote cell death remains to be elucidated. The fact that HIV-infected macrophages and/or T cells and cancer cells are more sensitive to autosis under some conditions is intriguing and suggests that induction of autosis may be utilized for selective elimination of certain cell populations.

Autosis has been reported in humans as well. Death of hepatocytes in patients with severe anorexia is accompanied by the presence of convoluted nuclei, electron-dense mitochondria, dilated ER, and numerous autophagic vacuoles and empty vacuoles, suggesting that these cells die by autosis (32). It would be interesting to test whether hepatic injury and decreased function in anorexia patients can be alleviated by cardiac glycosides. We recently showed that Tat-beclin 1 treatment induces autosis in human induced pluripotent stem cell-derived cardiomyocytes, suggesting that autosis can take place in human cells (23).

We expect that progress in the field will identify more examples of autosis in many other cell types under diverse conditions. It will be important to learn from the available examples when and how autosis is induced.
INDUCTION OF AUTOSIS IN THE HEART IN RESPONSE TO I/R

Because I/R strongly induces autophagy in the heart, it is natural to speculate that autosis is induced, as in the case of I/R injury, in the brain and kidney. In fact, we have shown recently that autosis of cardiomyocytes is induced in the heart in vivo in response to I/R (23). Here, we describe the key findings of this study and discuss the underlying mechanism of autosis induced by I/R. Autophagy is up-regulated by a short period of ischemia and further stimulated during reperfusion in the heart, whereas myocardial injury is attenuated when autophagy is suppressed by down-regulation of beclin 1 or other interventions (3). Cardiomyocytes located in the ischemic area exhibit accumulation of autophagosomes and autolysosomes, fragmented ER, electron-dense mitochondria, and mild chromatin condensation in response to reperfusion in a time-dependent manner. Cardiomyocytes start to show a characteristic PNS during the late phase of reperfusion, namely 6 h after reperfusion and thereafter, which is consistent with the induction of autosis. These morphological findings, which are consistent with autosis, and myocardial injury, as evaluated with 2,3,5-triphenyl tetrazolium chloride staining, in response to I/R, were significantly suppressed in the presence of ouabain treatment in humanized Na⁺,K⁺-ATPase knock-in mice, suggesting that I/R induces autosis, which contributes significantly to the overall I/R injury in the heart.

Interestingly, autosis is increased in the late phase of reperfusion. Although the consensus has been that the process of cell death in the ischemic area should be completed within 1 to 2 h of reperfusion, our results suggest that cardiomyocytes die continuously throughout the late phase of reperfusion via mechanisms distinct from apoptosis or necrosis. This provides a strong rationale for extending interventions to prevent reperfusion injury through the late phase of reperfusion.

MOLECULAR MECHANISMS OF AUTOSIS

Here, we discuss why strong activation of autophagy leads to autotic cell death (Figure 3). An important consideration is the fact that induction of autosis cannot be alleviated by lysosome inhibitors. Thus, autosis is not mediated through lysosomal degradation. Increasing lines of evidence suggest that excessive accumulation of autophagosomes may mediate autotic cell death. Excessive accumulation of autophagosomes can take place when the balance between synthesis and degradation of autophagosomes is disrupted. We will discuss this issue, using the induction of autosis in response to myocardial I/R as an example.

Although autophagic flux is up-regulated during the initial hours of myocardial reperfusion, it gradually decreases thereafter (23). We found that autosis is observed 6 h after reperfusion, when autophagic flux is attenuated. This appears counterintuitive because, by definition, autosis should be stimulated by autophagy. Importantly, however, marked accumulation of autophagosomes is observed during the late phase of reperfusion due to a high level of autophagosome formation despite decreased lysosomal degradation. Interestingly, rubicon, a molecule known to inhibit fusion between autophagosomes and lysosomes, is up-regulated during the late phase of reperfusion, thereby inhibiting autophagic flux. When autophagic flux is slowed down by rubicon, cardiomyocytes appear to try compensating for the reduced level of autophagic flux by making more autophagosomes. Thus, we speculate that up-regulation of rubicon dramatically stimulates accumulation of autophagosomes during the late phase of reperfusion. Cardiac-specific heterozygous down-regulation of rubicon restores autophagic flux and inhibits accumulation of autophagosomes, suggesting that rubicon plays an important role in inhibiting autophagic flux and, in turn, inducing accumulation of autophagosomes. Loss of rubicon function attenuates autosis and reduces myocardial infarct size, similar to the results observed in ouabain-treated humanized Na⁺,K⁺-ATPase knock-in mice. These results suggest that endogenous rubicon plays an essential role in mediating autosis during myocardial reperfusion. Although down-regulation of rubicon may reduce myocardial injury solely by improving autophagic flux, we believe that the consequent prevention of excessive autophagosome accumulation is also important because treatment with Tat-beclin 1, which stimulates autophagy, exacerbates autosis if it is given during the late phase of reperfusion (23).

Why is excessive accumulation of autophagosomes detrimental for cells? Because endomembranes are used as a membrane source for autophagosomes (33), excessive formation of autophagosomes may occur at the expense of intracellular organelle membranes, including ER, mitochondria, and even plasma membrane. Consistent with this hypothesis, activation of autosis in the heart is accompanied by decreases in intracellular membranes and a consequent reduction in the function of intracellular organelles, including depolarization of mitochondrial membrane potentials. On the other hand, down-regulation of vesicle-
associated membrane protein-associated proteins, which establish ER contact with autophagosome precursors, inhibits the consumption of ER membranes and even rescues Tat–beclin 1-induced autotic cell death (23). Importantly, whether decreases in intracellular membrane sources contribute to autotic cell death remains to be tested. To address this issue, it is necessary to investigate whether interventions to restore the endomembrane system level or intracellular organelles can rescue cells from autosis.

One of the most unique morphological features of autosis is the ballooning of the PNS. Previous reports showed a similar abnormal perinuclear morphology in human osteosarcoma U2OS cells, in the presence of overexpressed disease-associated mutant lamin B receptor (LBR) (34). LBR has sterol reductase activity, which is important for cholesterol biosynthesis (35). Expression of mutant LBRs and the related sterol reductases transmembrane 7 superfamily member 2 and 7-dehydrocholesterol reductase causes massive ER and PNS expansion, similar to the PNS in autosis. Because sterol content is not altered by overexpression of mutant LBRs, the sterol reductase activity may not be the direct trigger for the PNS-like structure. Alternatively, mutant LBRs or wild-type transmembrane 7 superfamily member 2 and 7-dehydrocholesterol reductase may change the membrane fluidity or permeability, thereby affecting osmolarity and signaling (34). Whether these mechanisms are involved in the pathogenesis of autosis remains to be elucidated.
Currently, the sensitivity to cardiac glycosides is among the most important clues to elucidating the molecular mechanism of autosis. Na\(^{+}\),K\(^{+}\)-ATPase physically interacts with beclin 1 to stimulate autosis. Cardiac glycosides disrupt the interaction between Na\(^{+}\),K\(^{+}\)-ATPase and beclin 1 by interacting with Na\(^{+}\),K\(^{+}\)-ATPase (22). It is possible that this interaction alters ion pump activity or ion exchange-dependent effects of Na\(^{+}\),K\(^{+}\)-ATPase. Na\(^{+}\),K\(^{+}\)-ATPase modulates calcium signaling through interaction with its steroid agonist ouabain (36). However, treatment of cardiomyocytes with a calcium chelator, 1,2-Bis(2-amino-phenoxo)ethane-N,N,N\(^{\prime}\),N\(^{\prime}\)-tetraacetic acid tetrakis(acetoxymethyl ester), did not affect autosis (23), suggesting that calcium may not be involved in autosis. Interestingly, the interaction between Na\(^{+}\),K\(^{+}\)-ATPase and beclin 1 takes place at many intracellular membranes, including ER, perinuclear membranes, mitochondria, and endosomes. Thus, the Na\(^{+}\),K\(^{+}\)-ATPase/beclin 1 interaction may trigger autosis through its effect on membrane ion, osmolyte, and fluid homeostasis across intracellular membranes (37). Further investigation is required to elucidate the role of the Na\(^{+}\),K\(^{+}\)-ATPase/beclin 1 interaction.

Another possibility is that Na\(^{+}\),K\(^{+}\)-ATPase alters the function of beclin 1, thereby affecting autophagic activity or vesicle trafficking. Because rubicon also physically interacts with beclin 1 (38), modulation of beclin 1 may play a key role in mediating autosis. However, the interaction between Na\(^{+}\),K\(^{+}\)-ATPase and beclin 1 alone may not affect the function of beclin 1 in such a way as to induce autophagy. Further investigation is required to clarify how the interaction between beclin 1 and either Na\(^{+}\),K\(^{+}\)-ATPase or rubicon affects the function and modulates the activity of autosis.

**CLINICAL IMPLICATIONS**

Acute myocardial infarction is among the main causes of morbidity worldwide (39). Preventing death of cardiomyocytes is a major goal in the treatment of cardiac disease. Unfortunately, however, medical treatments targeting any single form of cell death in cardiomyocytes do not appear to be significantly effective for alleviating cardiac dysfunction in many clinical conditions (40). A possible reason for this could be that cardiomyocytes die by many mechanisms, with different time courses in response to different forms of stress. Thus, it is important to determine specifically how cardiomyocytes die in the presence of a given stress and clarify the time course of death. For example, the fact that autosis is observed during the late phase of I/R is unexpected in that it has generally been believed that death of cardiomyocytes takes place within the first few hours of reperfusion; thus, it has never been targeted. However, this might partially explain why a therapy focusing on either apoptosis or necrosis alone may not be able to reduce reperfusion injury. If autosis continuously kills cardiomyocytes during the subacute phase of I/R injury, it might prove useful to add a treatment targeting autosis to other forms of treatment targeting apoptosis and necrosis, which primarily take place during the early phase of I/R.

Because autosis is induced by signaling mechanisms molecularly distinct from those of apoptosis and necrosis, treatment targeting autosis should show additive effects to those targeting other forms of cell death.

The human body produces endogenous cardiac glycoside-like substances. Inhibiting endogenous cardiac glycoside-like substances with DigiFab (BTG International Inc., West Conshohocken, Pennsylvania) exacerbates cardiac autosis during exercise (22), suggesting that endogenous cardiac glycoside-like substances have the ability to limit autosis during stress. Thus, if one could find an intrinsic mechanism to up-regulate endogenous cardiac glycosides, stimulation of this endogenous mechanism might prevent autosis and myocardial injury during I/R. Given that cardiac glycosides have the general side effect of inducing cardiac arrhythmia, however, it would be essential to develop a safer intervention to inhibit autosis.

Currently, other cardiac conditions besides I/R injury where autosis contributes to cardiomyocyte death and either myocardial injury or heart failure remain to be identified. For example, the level of autophagy is dramatically increased 3 to 4 days after permanent coronary ligation, during the acute phase of pressure overload or in response to doxorubicin-induced cardiomyopathy (4,41). In these conditions, suppression of autophagy effectively reduces myocardial cell death and improves either left ventricular function or the survival of the animals (4,42) (Table 2). Marked accumulation of autophagosomes in the heart is also observed in lysosomal storage disease (43). It would be important to investigate whether autosis is involved in any of these cardiac conditions and, if so, to test whether interventions to block autotic cell death can alleviate cardiac injury or cardiac dysfunction. It should be noted that the
molecular mechanisms through which excessive autophagy induces cell death and, consequently, interventions to alleviate cell death induced by autophagy in these cardiac conditions may not be identical. For example, inhibition of rubicon may not be effective when a genetic defect responsible for a lysosomal disease causes an irreversible defect in lysosomal function.

If depletion of intracellular membranes is a key facilitator of autosis, molecular interventions to facilitate generation of the endomembrane system during autosis. For example, testing whether facilitating the production of membrane phospholipid can restore cellular function and prevent cell death would be of great interest.

In summary, autosis is an attractive therapeutic target in cardiac disease because it is activated through unique signaling mechanisms with a distinct time course compared with other forms of cell death. Thus, one could expect additive effects when autosis inhibitors are applied in conjunction with existing modalities of treatment. Further investigation should be focused on identification of medical conditions in which autosis is activated and the elucidation of underlying signaling mechanisms.

**CONCLUDING REMARKS**

There is increasing evidence that cardiomyocytes can die through autosis under some conditions. This suggests that it may be possible to reduce the extent of cardiomyocyte death and myocardial injury in some conditions by targeting autosis. Even if autophagy is initially activated as an adaptive mechanism, it can become maladaptive in a time- and dose-dependent manner. Thus, if autosis is targeted for treatment of myocardial injury, it is important to correctly evaluate the extent of autophagy and identify the morphological and

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**TABLE 2 Examples of Maladaptive Autophagy in the Heart**

| Insult | Model | Autophagy Modulator | Autophagy Activity | Result | Ref. # |
|--------|-------|---------------------|--------------------|--------|--------|
| I/R    | Adult mouse | Beclin 1<sup>+/–</sup> mouse | Autophagy is drastically activated during I/R in the heart | Reduced myocardial infarct size with reduced autophagy in beclin 1<sup>+/–</sup> mice | Matsui et al. (3) |
| I/R    | Rubicon<sup>+/–</sup> mouse | Autophagy is drastically activated during I/R in the heart | Myocardial infarct size is reduced by restoration of autophagic flux in rubicon<sup>+/–</sup> mice during I/R | Nah et al. (23) |
| I/R    | LncRNA CAIF | LncRNA CAIF directly binds to p53 and inhibits p53-mediated autophagy activation during I/R | Knocdown of miR-30 by inhibiting autophagy | Liu et al. (44) |
| I/R    | ALDH2 | ALDH2 activates autophagy during ischemic reperfusion | Overexpression of ALDH2 rescues myocardial injury by inhibiting autophagy during reperfusion | Ma et al. (45) |
| H/R    | Neonatal rat CMs | Overexpression or knockdown of beclin 1 | Autophagosome clearance is impaired during I/R and H/R | Beclin 1 knockdown restores autophagosome processing and attenuates cell death by H/R | Ma et al. (46) |
| H/R    | DOX-induced cardiomyopathy | Beclin 1<sup>+/–</sup> mouse | DOX blocks autophagic flux in the heart | Beclin 1 knockdown restores autophagosome processing and attenuates cell death by H/R | Li et al. (42) |
| H/R    | Cardiac hypertrophy | HDAC inhibitors | HDAC inhibitors attenuate autophagy during TAC | HDAC inhibitors alleviate TAC-induced hypertrophy by inhibiting autophagy | Cao et al. (47) |
| Diabetic cardiomyopathy | Adult mouse | Beclin 1<sup>+/–</sup> mouse | Autophagy activity is reduced in type 1 diabetic heart | Further reduction in autophagy by beclin 1<sup>+/–</sup> protects the heart against diabetic cardiomyopathy | Xu et al. (19) |
| Arrhythmia | Neonatal rat CMs | 3-MA, shAtg7, and shbeclin 1 | Hyperglycemia reduces autophagic flux | Additional suppression of autophagy by 3-MA or shAtg7 and shbeclin 1 attenuates high glucose-induced CM death | Kobayashi et al. (49) |
| Arrhythmia | Rabbit | Lentivirus-mediated Atg7 knockdown or (CQ) | Autophagic flux is markedly activated in AF patient and rabbit model | Atg7 knockdown or CQ restored the shortened atrial effective refractory period and alleviates the AF vulnerability by inhibiting autophagy in rabbit | Yuan et al. (50) |

3-MA = 3-methyladename; AF = atrial fibrillation; ALDH2 = aldehyde dehydrogenase 2; Atg7 = autophagy-related protein 7; CAIF = cardiac autophagy inhibiting factor; CM = cardiomyocyte; CQ = chloroquine; DOX = doxorubicin; GATA4 = GATA binding protein 4; HDAC = histone deacetylase; H/R = hypoxia-reoxygenation; I/R = ischemia-reperfusion; LncRNA = long noncoding ribonucleic acid; miR-30 = microRNA-30; sh = shRNA; TAC = transverse aortic constriction.
biochemical features of autosis to confirm that autophagy is no longer protective in a given condition. To date, several cardiac conditions have been shown to exhibit very strong autophagy and down-regulation of autophagy protects the heart in such conditions. It will be interesting to test whether autosis is observed in these conditions and, if so, whether cardiac glycosides can reduce cardiomyocyte death and improve cardiac function. If autotic cell death is identified in a given condition, it would be important to investigate how autophagy becomes dysregulated in that condition and how autosis kills cardiomyocytes. Apoptosis can be an orderly process and, thus, is physiological under some conditions. It remains unclear whether cardiomyocytes use autosis as a salutary mechanism. Because marked accumulation of autophagosomes is often accompanied by a mismatch between autophagosome formation and lysosomal degradation, how the mismatch takes place and amplifies the accumulation of autophagosomes should be clarified. Because beclin 1-interacting proteins, including Na<sup>+</sup>,K<sup>+</sup>-ATPase and cardiac glycosides, have been shown to be involved in autosis, it will be interesting to further investigate how beclin 1, rubicon, Na<sup>+</sup>,K<sup>+</sup>-ATPase, and possibly other interacting proteins induce cell death. By conducting a genome-wide small, interfering ribonucleic acid screen, Fernandez et al. (22) identified potential mediators of autosis besides Na<sup>+</sup>,K<sup>+</sup>-ATPase, including some involved in ion transport and cell-to-matrix adhesion. Further investigation to determine how those molecules participate in autosis would clarify the molecular mechanism of autosis. It would also be interesting to test whether chemical inhibitors against the molecules identified by the small, interfering ribonucleic acid screen can inhibit cardiac autosis in response to I/R. Because disappearance of intracellular organelles is an important feature of autosis and is likely to induce cellular malfunction, molecular mechanisms through which dysregulation of autophagosome formation leads to the disappearance of intracellular organelles should also be investigated. Elucidation of the underlying molecular mechanism of autosis should provide multiple options for identifying the occurrence more easily and conveniently and, eventually, should allow for the development of novel and selective interventions to treat cardiac disease. Because specific therapy for autosis has never been combined with therapies for other forms of cell death and because autosis occurs with a time course different from that of other forms of cell death, the development of a specific therapy for autosis could potentially represent a breakthrough in the treatment of myocardial I/R injury. Thus, further investigation regarding autosis in the heart is warranted.

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