AWARD NUMBER: W81XWH-16-1-0244

TITLE: Epicatechin as a therapeutic strategy to mitigate the development of cardiac remodeling and fibrosis

PRINCIPAL INVESTIGATOR: Francisco Villarreal

CONTRACTING ORGANIZATION: University of California, San Diego
La Jolla, CA 92093

REPORT DATE: Sept 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Sept 2018	Annual	10 Aug 2017- 9 Aug 2018

4. TITLE AND SUBTITLE

Epicatechin as a therapeutic strategy to mitigate the development of cardiac remodeling and fibrosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0244

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Francisco Villarreal

E-Mail: fvillarr@ucsd.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Diego
9500 Gilman Dr. Dept. 621
La Jolla, CA 92093-0621

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Heart failure with preserved ejection fraction (HFpEF) continues to increase and little is known about its pathophysiology. About 2/3 of patients are women and risk factors include aging, hypertension and metabolic syndrome. A feature of the disease is cardiac fibrosis. Currently, no drugs target HFpEF and the development of animal models can assist in therapy evaluation. We developed a female rat model of aging, estrogen depletion and metabolic syndrome to evaluate the role of these factors in altering cardiac structure/function. Aged female Fischer F344 rats were allocated into an aging group, aging + ovariectomy and aging + ovariectomy + 10% fructose in drinking water. At 22 months of age, animals were anesthetized and left ventricular (LV) function was evaluated. Histological measures were also obtained. Intraventricular pressure-volume loop analysis evidenced significant decreases in stroke work cardiac output and increases in myocardial stiffness with ovariectomy. Histological analysis indicated increasing levels of inflammatory infiltration, perivascular and interstitial fibrosis with ovariectomy and with fructose supplementation. In conclusion, with aging, estrogen deprivation, markedly deteriorates myocardial microstructure which may facilitate the loss of diastolic and systolic function. This model may serve to understand the role that aging and menopause may have in the development of HFpEF.

15. SUBJECT TERMS
fibrosis, myocardium, heart failure, stiffness, estrogen, aging, metabolic syndrome

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	18	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

The project proposes that treatment with the flavanol (-)-epicatechin (Epi) will ameliorate adverse tissue remodeling and cardiac fibrosis in female animal models developing diastolic dysfunction as seen in women with heart failure with preserved ejection fraction (HFpEF). The project's 3 specific aims are (1) to determine if early use of Epi in female animal models of fibrotic hearts will reduce collagen deposition and preserve function, (2) to determine if late use of Epi in female animal models of fibrotic hearts will reduce collagen deposition and recover function, and (3) to investigate if the beneficial effects of Epi are due to its action on the cardiac fibroblast which are the cells mainly responsible for the production of fibrillar collagens. The mechanism(s) and functional outcomes of oral Epi preventive and therapeutic treatments will be defined in a relevant female animal model of diastolic dysfunction and can potentially lead to the design and implementation of clinical trials for the treatment for myocardial fibrosis leading to improved function.

KEYWORDS

Fibrosis, myocardium, heart failure, aging, estrogen, metabolic syndrome, stiffness, collagen, compliance, remodeling, epicatechin, flavanols, left ventricle.

ACCOMPLISHMENTS

I. Major goals of the project

Major Goals (Aims): The following are the **major tasks** identified in the Statement of Work associated with each aim.

Aim 1 related: Early preventive treatment with (-)-epicatechin (Epi) prevents myocardial fibrosis

1. Characterize effects of aging on myocardial fibrosis in untreated animals (90% completed)
2. Characterize effects of early Epi treatment on myocardial fibrosis in a model of estrogen depletion and aging (65% completed)
3. Characterize effects of early Epi treatment on myocardial fibrosis in a model of estrogen depletion, aging and fructose supplementation (65% completed)

Aim 2 related: Late treatment with Epi reverses myocardial fibrosis

4. Characterize long-term baseline effects of aging on myocardial fibrosis (90% completed)
5. Characterize the reversal of myocardial fibrosis by late Epi treatment in a model of estrogen depletion and aging (75% completed)
6. Characterize effects of estrogen depletion, aging and fructose supplementation on myocardial fibrosis and its reversal by late Epi treatment (75% completed)

Aim 3 related: The anti-fibrotic effects of (-)-epicatechin are mediated by TGF-β1 inhibition

7. Cardiac fibroblast phenotype characterization (30% completed)
8. Effects of profibrotic phenotype stimulation/inhibition (20% completed)
9. Gene expression modulation (pending)

II. Accomplishments

Aim 1 related

A. *Characterizing the effects of aging, estrogen depletion and excess weight on left ventricular (LV) remodeling*

During year 2, a large part of the project's effort related to completing the implementation and assessment of the female model of aging driven cardiac remodeling and fibrosis that was to be compounded by ovariectomy (estrogen depletion) and fructose supplementation (yielding excess weight gain). Over the course of the last few months, we have finished the characterization of changes in cardiac structure/function that develop as a function of aging (alone), aging + ovariectomy and aging + ovariectomy + fructose supplementation. A highly detailed and extensive characterization of the models has been submitted for publication (see bibliography list). We rate the achievement level at ~90%, as we have now proceeded to use the data gathered from these in vivo studies to implement computer models that will allow use to simulate the impact that structural changes have on diastolic function (mechanical properties).

As listed in the proposed project plans, we completed the following tasks/subtasks:

- Assessed serial changes in cardiac structure/function using echocardiography
- Implemented terminal studies and recorded detailed carotid and intraventricular hemodynamics
- Performed ex vivo pressure-volume curves to examine changes in global left ventricular (LV) compliance
- Performed ex vivo pressure-strain curves to examine changes in free wall LV epicardial strains
- Fixed hearts, measured and recorded detailed histomorphometric parameters
- Compiled and summarized all data and performed a rigorous statistical evaluation, wrote reports and shared data

Methods utilized include echocardiography to measure in vivo changes in heart morphology, diastolic and systolic function. In vivo carotid and LV hemodynamics utilized a Millar pressure conductance catheter. At the time of the terminal study, an ex vivo assessment of LV pressure volume and strain was implemented using an inflatable balloon as well as video recording of the inflating hearts to monitor the displacement of epicardial markers and calculate two-dimensional strains. Finally, hearts were fixed and sectioned for detailed histological analysis using hematoxilin and eosin and Sirius Red staining. Once stained, sections were visualized using standard microscopy and images analyzed for LV morphometry and collagen density by using a high resolution digital system (HALO system). All data was summarized in databases and subjected to rigorous statistical analysis using GraphPad software.

Aim 1 results have led to the submission of a research article to the *J of the Am Coll Cardiol, Basic to Translational Science*. The following is the abstract of the submitted manuscript:

Unmasking of Estrogen Dependent Left Ventricular Dysfunction in Aged Female Rats: A Potential Model of Early Stage HFpEF

Background: The incidence of heart failure with preserved ejection fraction (HFpEF) has increased and its pathophysiology is unknown. Two-thirds of HFpEF patients are older women and risk factors include hypertension and metabolic syndrome. The study goal was to evaluate the roles of aging, estrogen depletion and excess weight on altering cardiac structure/function.
Methods: We implemented female animal models of aging, estrogen depletion and metabolic syndrome. Female, 18 month old, Fischer F344 rats were divided into aging group, aging + ovariectomy (OVX) and aging + ovariectomy plus 10% fructose (OVF) in drinking water (n=8-16/group). Left ventricular (LV) structure/function was monitored by echocardiography. At 22 months of age, animals were anesthetized and catheter-based LV function evaluated, followed by histological measures of chamber morphometry and collagen density.
Results: OVF animals increased body weight. While echocardiography did not detect major alterations, intraventricular pressure-volume loop analysis showed significant ($p<0.05$) decreases (vs. aging) in stroke volume (13% OVX and 15% for OVF), stroke work (34% and 52%), cardiac output (29% and 27%), and increases in relaxation time (10% OVX) and LV end-diastolic pressure volume relationship slope with ovariectomy (34% OVX) with preserved ejection fraction. Histological analysis indicated interstitial fibrosis with aging, which was higher in the endocardium of OVX and OVF groups.
Conclusion: In the setting of aging, ovariectomy leads to the loss of diastolic and global LV function in the presence of preserved ejection fraction. This model may serve to understand the roles that aging and estrogen depletion have in the development of HFpEF. [**end of abstract**].

The following sections highlight significant results generated that are reported in greater detail in the complete manuscript:

General parameters

Total body weight and percent body weight gain increased with aging and OVX demonstrating a 6% and 10% increase in weight whereas the OVF group gained 26%. There were no differences in heart weights between aged groups, but there was a difference between OVF vs. young ($p<0.05$ by unpaired t-test). Calculated body surface area was higher in OVF and OVX vs. aged ($p<0.001$).

Echocardiography

As shown in figure 1, echocardiographic results reveal a time-dependent effect on cardiac morphometry in aged, OVX and OVF groups. Anterior wall thickness in diastole (AWThD) significantly decreased as a function of time with no differences between groups (panel A). With wall thickness during systole (AWThS), there was a significant difference between aged vs. OVX and OVF. For posterior wall diastolic and systolic (PWThD and PWThS) there was also a decrease in thickness over time without differences between groups (B). There was a significant time dependent increase in chamber diameters (C, D) in all groups. Heart rate (HR), ejection fraction (EF) and fractional shortening (FS) were also not different between aged, OVX and OVF groups (data not shown). All of the above noted parameters were different vs. young animals.

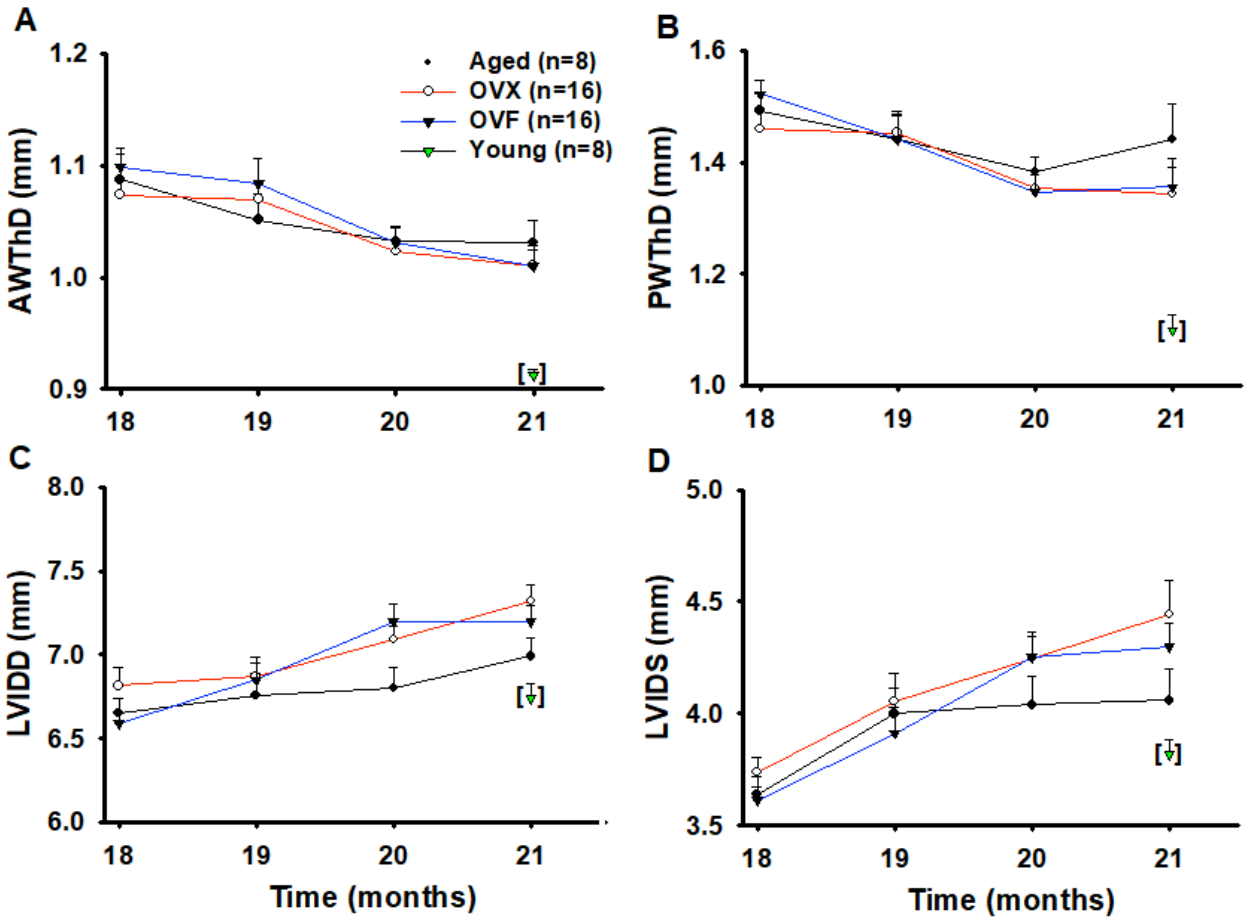

Figure 1. Left ventricular (LV) remodeling as serially tracked by echocardiography. Young group single time-point data included as reference. (A) Anterior wall thickness in diastole (AWThD). (B) Posterior wall thickness in diastole (PWThD). (C) LV internal diameter diastole (LVIDD). (D) LV internal diameter in systole (LVIDS). For panels A, B, C and D, $p<0.001$ for time dependent changes in all groups. OVX: ovariectomized, OVF: ovariectomized + fructose. Values are mean ± SEM.

Hemodynamic measurements

As shown in figure 2, for systolic aortic pressure (Pao) there was a significant increase in aged groups vs. young. Cardiac index (output normalized to body weight) was decreased in aged, OVX and OVF vs. young animals while OVX and OVF were different vs. aged. Stroke volume index was reduced in aged, OVX and OVF vs. young, and differences between OVX and OVF were also present vs. aged indicating an effect of ovariectomy and fructose on cardiac function. Ejection fraction was stable and not different between groups, being preserved at the normal range of ≥50% (D). Figure 3 depicts isovolumic relaxation time constant (IVRT) also known as "Tau" and arterial elastance (Ea) respectively. An increase in Tau was noted in aged, OVX and OVF groups vs. young. Arterial elastance was significantly elevated in OVX and OVF vs. young with a trend for an increase in the aged group.

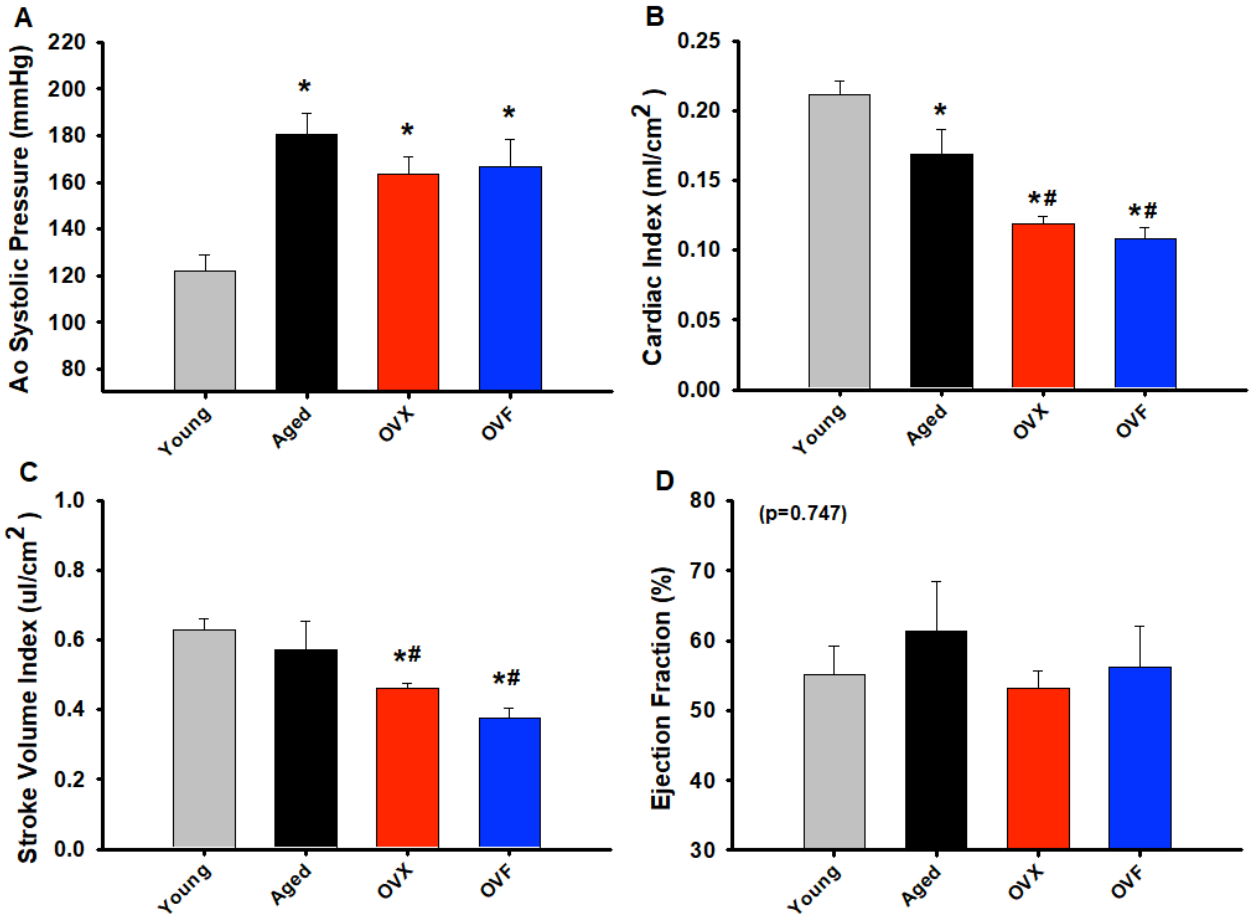

Figure 2. Hemodynamic values derived from arterial and left ventricular (LV) conductance catheter measurements during the terminal study. (A) Systolic aortic pressure ($p<0.05$ aged, OVX and OVF vs. young). (B) Cardiac index (*$p<0.05$, aged, OVX and OVF vs. young; and $^{\#}p<0.05$, OVX and OVF vs aged). (C) Stroke volume index ($p<0.05$ OVX and OVF vs young, and $^{\#}p<0.05$, OVX and OVF vs aged). (D) Ejection fraction. OVX: ovariectomized; OVF: ovariectomized + fructose. Values are mean ± SEM.

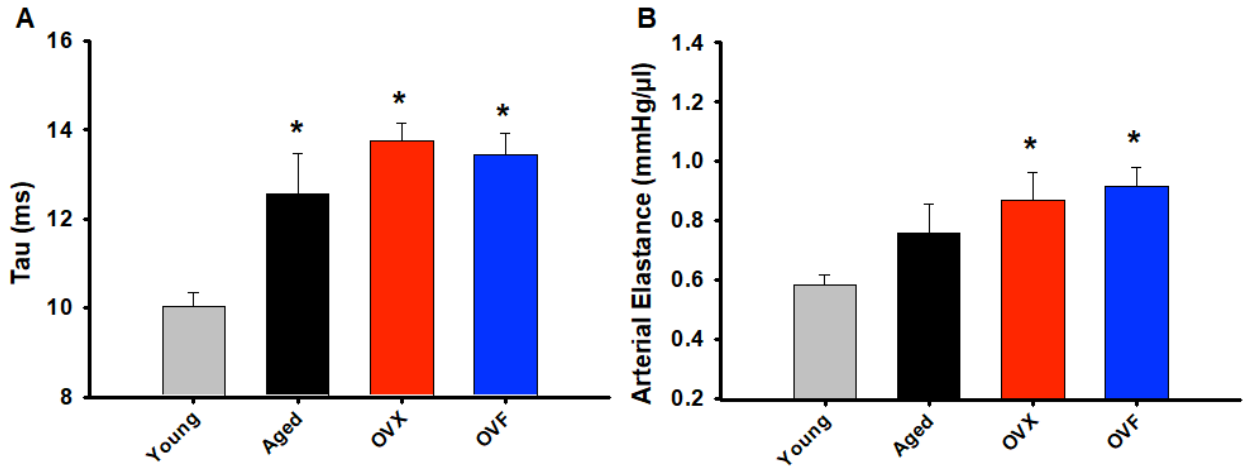

Figure 3. Hemodynamic values derived from arterial and left ventricular (LV) conductance catheter measurements during the terminal study. (A) LV isovolumic relaxation time constant (Tau) ($p<0.05$ aged, OVX and OVF vs. young). (B) Arterial elastance ($p<0.05$ OVX and OVF vs. young). OVX: ovariectomized; OVF: ovariectomized + fructose. Values are mean ± SEM.

Ex-vivo LV mechanics

As shown in figure 4, the analysis of LV PV curves did not demonstrate differences among the aged, OVX and OVF groups. However, all these groups were different vs. young demonstrating a global right-shift in aged, OVX and OVF animals. Epicardial circumferential strain analysis revealed no differences in E_{11} between aged, OVX and OVF groups or vs. young. In longitudinal strain, E_{22} there was a significant decrease in OVX vs. young ($p<0.001$). Overall, E_{11} strains were higher than E_{22} for all groups, where the range for E_{11} was 0.06-0.08 (i.e. 6-8 %) and for E_{22} 0.02-0.04.

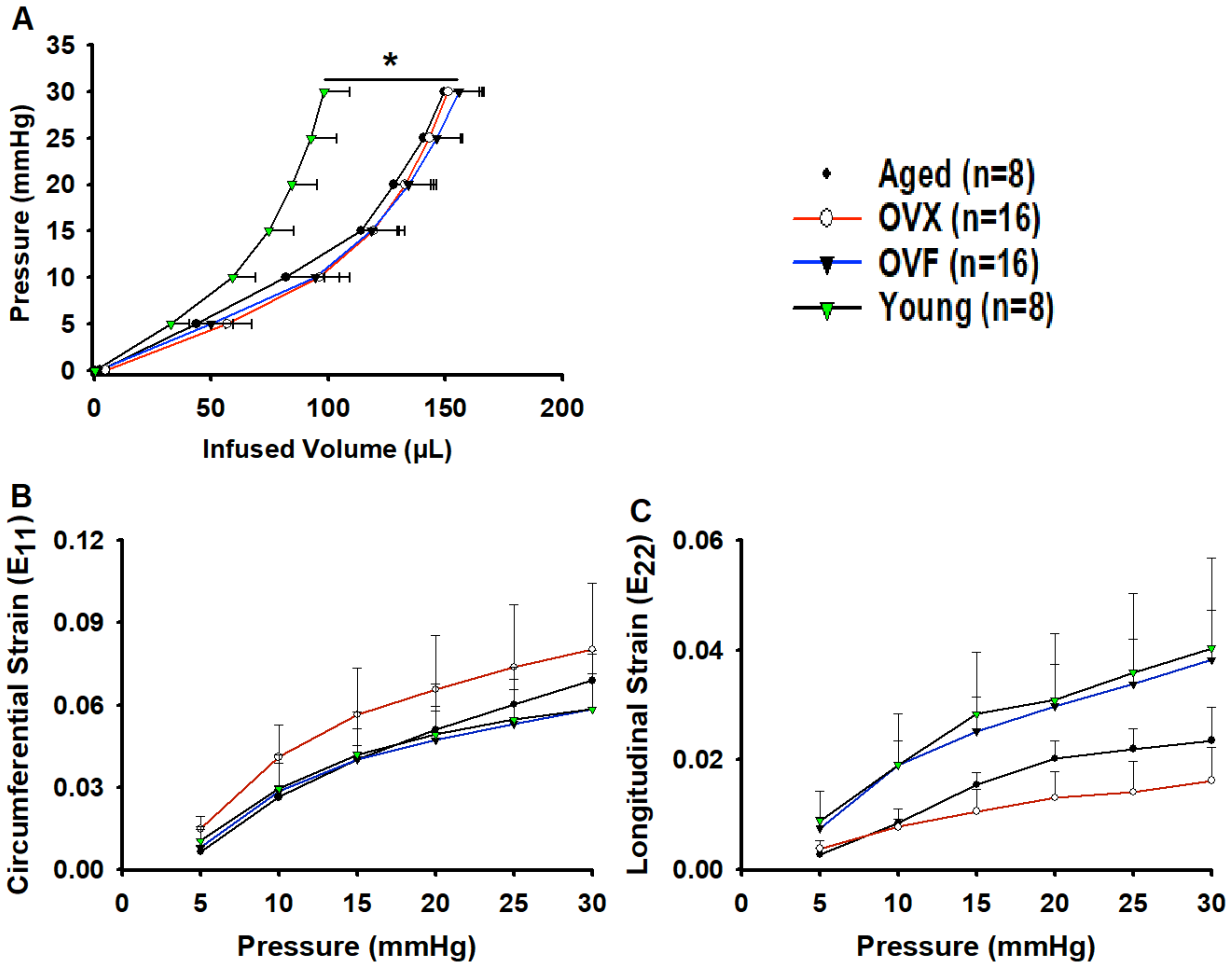

Figure 4. Ex-vivo analysis of left ventricular (LV) mechanics. (A) Passive LV pressure-volume (PV) curves for all groups at 21 months of age ($p<0.001$ aged, OVX and OVF vs. young). (B and C) Two-dimensional circumferential (E_{11}) and longitudinal (E_{22}) LV epicardial strains at incremental LV pressures ($p<0.001$, OVX vs. young for longitudinal strain). OVX: ovariectomized; OVF: ovariectomized + fructose. Values are mean ± SEM.

Histological analysis and collagen quantification

Representative Sirius Red stained cross-sections of hearts from select animals of the different groups at low and high magnifications are shown in figure 5. Images similar to those shown in the panel were used to quantify collagen abundance in the LV by segments (12 free wall and 6 septal). Interestingly, a visual inspection of large areas of abnormal (patch like) fibrosis present in papillary muscles indicated that aged hearts showed such lesions in 1/4 aged animals, 4/6 OVX and 5/7 OVF vs. none in young animals. Results from morphometry and histology are

summarized in figure 6. Aged rats demonstrated a higher LV tissue area vs. young ($p<0.05$). Aged, OVX and OVF groups exhibited increased LV collagen area, % free wall, septal and total LV collagen vs. young. Whereas the endocardial/epicardial ratio did not demonstrate significant overall differences by ANOVA among the groups, there were differences between OVX and OVF vs. young (by unpaired t-test).

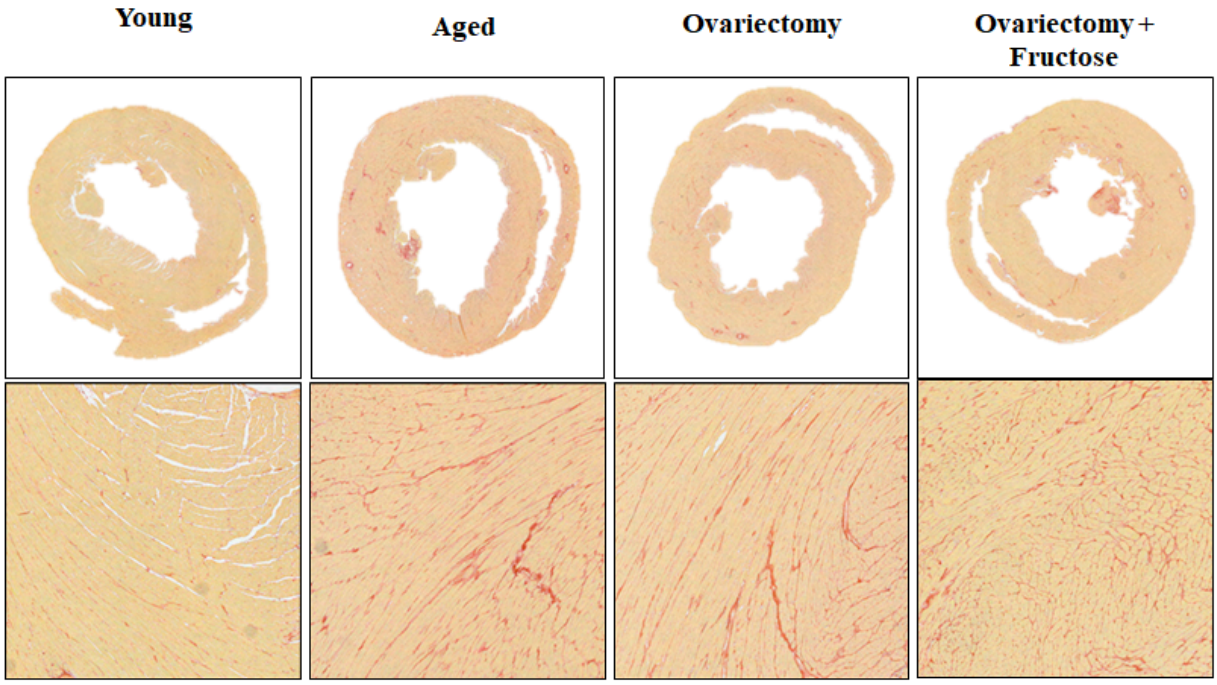

Figure 5. Representative images 2 and 20 X magnification from Sirius Red staining of hearts from all groups.

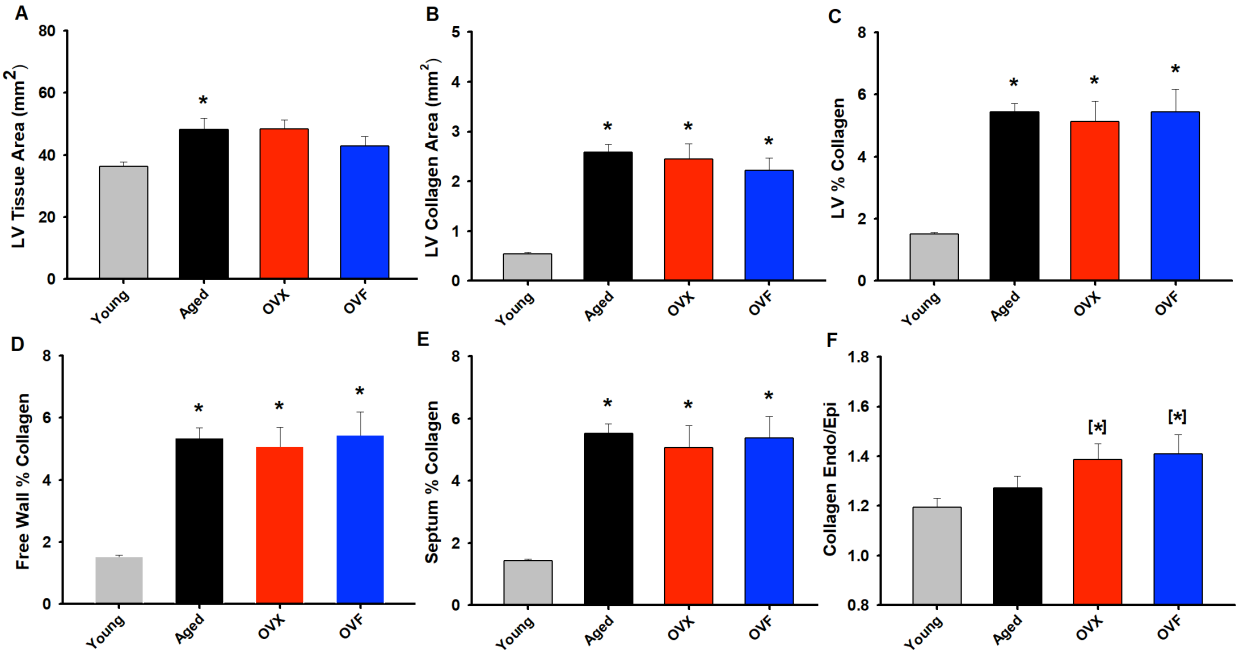

Figure 6. Histomorphometric analysis of LV tissue sections. (A) LV tissue area, (B) LV collagen area, (C) LV % collagen (collagen/LV area), (D) Free wall % collagen, (E) Septum % collagen, (F) Endocardium/epicardium collagen ratio. Values are mean ± SEM. *$p<0.05$ vs young, ANOVA. [*] $p<0.05$ vs young, unpaired t-test.

Major findings from this study can be summarized as follows:

- As expected, aged animals supplemented with fructose demonstrated a weight gain sufficient to substantiate as excess weight (>20%) vs. other groups
- As per echo results, all three groups developed slowly over time and in a similar magnitude, eccentric LV remodeling (i.e. chamber dilation)
- In all three aged groups, there is a prolongation of the half-time for diastolic relaxation and increases in arterial elastance with low estrogen levels and excess weight
- With aging, female rats develop systolic hypertension
- With aging, female rats demonstrate a loss of systolic function (cardiac output/index) which is further aggravated by the loss of estrogens and excess weight
- This loss of cardiac output occurs in the presence of normal ejection fraction
- With aging, an increase in interstitial collagen is noted which becomes greater in the endocardium of low estrogen and excess weight rats
- An increase in the presence of papillary fibrosis occurs in rats that is more evident with low estrogen and excess weight

Conclusions and Clinical Perspectives

On the basis of the detrimental changes noted in LV structure/function due to aging with estrogen depletion in the setting of preserved EF, it can be argued that this model may be useful in examining the role that these combined factors may play in the early stage development of HFpEF. These changes likely would be further accentuated if animals were allowed to survive for a longer period of time. Surprisingly, the development of interstitial fibrosis as a function of aging (about double vs. young) did not alter the mechanical properties of the LV. However, an increase in Tau suggests impaired "active" relaxation, possibly related to alterations in calcium handling. The loss noted in stroke volume (and consequently in cardiac output) in ovariectomy does indicate that estrogen depletion can notably contribute to impaired pump function. These data suggest that the use of invasive methods may be required to accurately recreate HFpEF in animal models. It is worth noting that all measurements in the current studies were done in a "resting" state. The true unmasking of the effect that specific interventions have in aged models may require the use of "stress" tests such as dobutamine infusions during the acquisition of LV hemodynamics. This modality of testing would be equivalent to that done in HFpEF patients to truly unmask the degree of LV dysfunction.

As HFpEF is most frequently seen in women, its natural history suggests a possible early role for aging, estrogens and/or risk factors such as hypertension. As the pathophysiology of HFpEF remains unclear, the identification of early stages of the disease is critical to its understanding. However, it is difficult to envision how this goal can be met in a typical patient population since the disease likely evolves slowly over time. Thus, the need to develop and implement animal models that mimic early stages of the disease and would allow for the evaluation of novel therapeutic strategies targeting those elements identified as critical to its pathophysiology.

B. *Characterizing the effects of early Epi treatment on aging, estrogen depletion and excess weight associated LV remodeling/fibrosis*

During year 2 of the project, we have implemented studies to assess the effects of early Epi treatment on aging associated cardiac remodeling in the absence or presence of estrogens and

excess weight as per fructose supplementation. We have completed ~65% of the project as in vivo studies are still ongoing. No major issues have arisen and these studies should be completed over the next 3-4 months including all physiological and histological analyses such that clear conclusions can be derived from the effects of early Epi treatment.

Aim 2 related

Characterizing the effects of late Epi treatment on aging, estrogen depletion and excess weight associated LV remodeling/fibrosis

During the last year, we completed all of the in vivo studies assessing the effects of late Epi treatment on aging associated cardiac remodeling in the absence or presence of estrogens and excess weight. All data is in the process of being compiled into databases and analyzed for statistical significance. Preliminary analysis of histological measures of collagen area fraction indicate an anti-fibrotic effect of Epi treatment. Figure 7 is partial data derived from the analysis of hearts indicating the apparent suppression of aging associated cardiac fibrosis. As results are compiled and analyzed, we will be able to determine if late Epi treatment also impacts the LV physiological measures recorded.

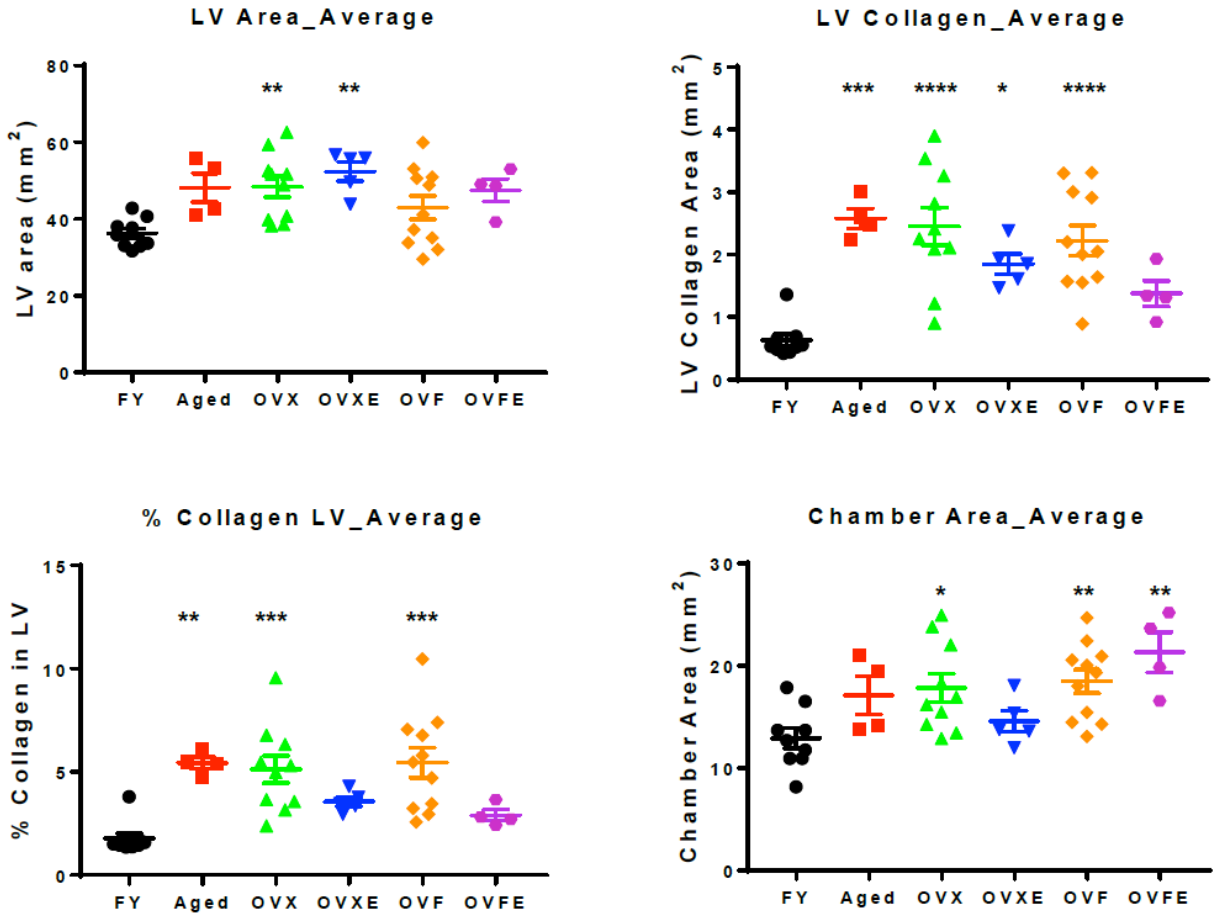

Figure 7. Histomorphometric analysis of LV tissue sections. Partial results are summarized for LV tissue area, collagen area, % collagen (collagen/LV area), and LV chamber area. Values are mean ± SEM. *p<0.05 vs young, ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. young by ANOVA. OVXE and OVFE = overiectomy and ovariectomy + fructose treated with Epi.

Aim 1 and 2 related: Mathematical modeling studies

Fibrillar collagen plays a major role in the passive material properties of the myocardium. Hearts with severe fibrosis have significantly higher tissue stiffness, which can affect overall filling mechanics of the ventricular chamber. It has been shown that cardiac fibrosis is not a homogenous process, and we are employing modeling approaches of diastolic ventricular function to examine the significance of these regional differences in fibrosis. Many studies have modeled infarct zones using finite-element methods, generally by drastically increasing the stiffness of the infarct scar and border zone. To model the pathophysiology of cardiac fibrosis, a similar approach was used in preliminary studies to model rat LV during passive inflation. Utilizing realistic ventricular geometry, muscle fiber structure and collagen distribution, the goals of the model include quantifying the local material properties, deformations and stresses, and overall passive filling function. Passive material properties are important to diastolic function, and can be used to quantify the efficacy of treatments for heart failure in animal models. A transverse isotropic constitutive law with respect to the local fiber axis was used for this initial model (figure 8). Experimental results from aim 1 studies showed increased collagen area fraction in the subendocardium vs. epicardium. Simulations using a bulk measurement of collagen versus a transmural variation were compared. These preliminary results show only modest differences between models with a single material vs. those with transmurally varying properties. Further studies will determine these differences in a subject-specific fashion in which we expect to see larger effects of the fibrosis gradients in particular if one assumes that as animals become even older fibrosis continues to increase with greater levels in endocardium. We will also compare local stresses and deformations between groups and methods, which may unmask the functional effects of heterogeneous fibrosis in these models.

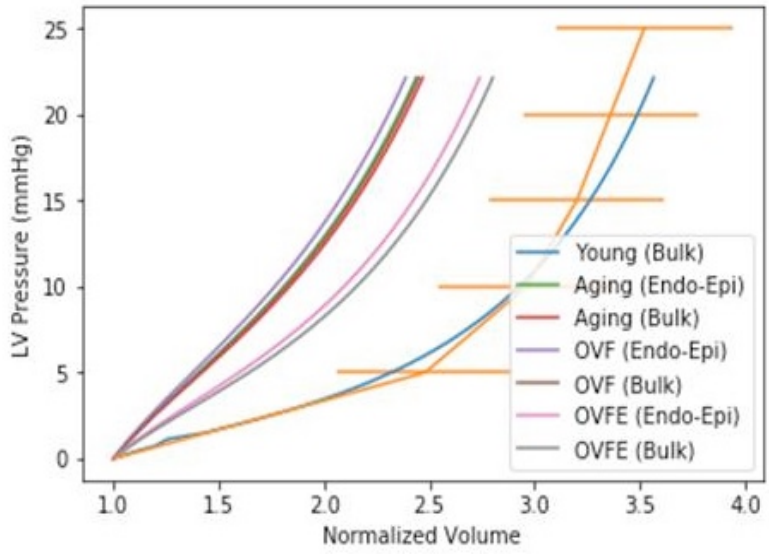

Figure 8. Passive pressure-volume curves predicted from a geometric model fit to typical control (young) experimental data (shown in yellow with error bars), comparing hearts with varying material properties. Fibrosis was modeled as a stiffness parameter based on the measured collagen area fractions in aging, ovariectomy + fructose (OVF) and OVF + epicatechin treatment (OVFE). OVFE shows expected changes in chamber compliance, but models with transmural differences (Endo-Epi) do not differ from models using a single (Bulk) material law.

Aim 3 related

The anti-fibrotic effects of (-)-epicatechin are mediated by TGF-β1 inhibition

Over the course of the last year and a half, we have implemented the culturing of cardiac fibroblasts isolated from young and aged female hearts. Interestingly, there is little precedent in this regard and initially we detected a lower yield of cells vs. male hearts. We have adjusted and optimized tissue culture conditions, which have led to reasonable yields and culturing of cells. In attempting to challenge the cells to develop a pro-fibrotic phenotype using high glucose media or angiotensin II, we have noted a limited responsiveness to the stimulation vs. our historical strong response from male derived cells. On these basis, we are now utilizing cell culture media that is essentially devoid of estrogens as we believe their effect is likely protecting the cells from pro-fibrotic stimuli (in fact, the very limited published reports suggest this is the case). Furthermore, we are also using cell media devoid of phenol as this dye can stimulate estrogen receptors.

In figure 9 below, we summarize Western blot generated preliminary data from experiments in which female cardiac fibroblasts obtained from young hearts were stimulated with high glucose media for 48 h triggering the excess enzymatic glycosylation of total proteins (as per RL2 antibody signal), increases in TGF-β1 and the associated Smad signaling pathway levels.

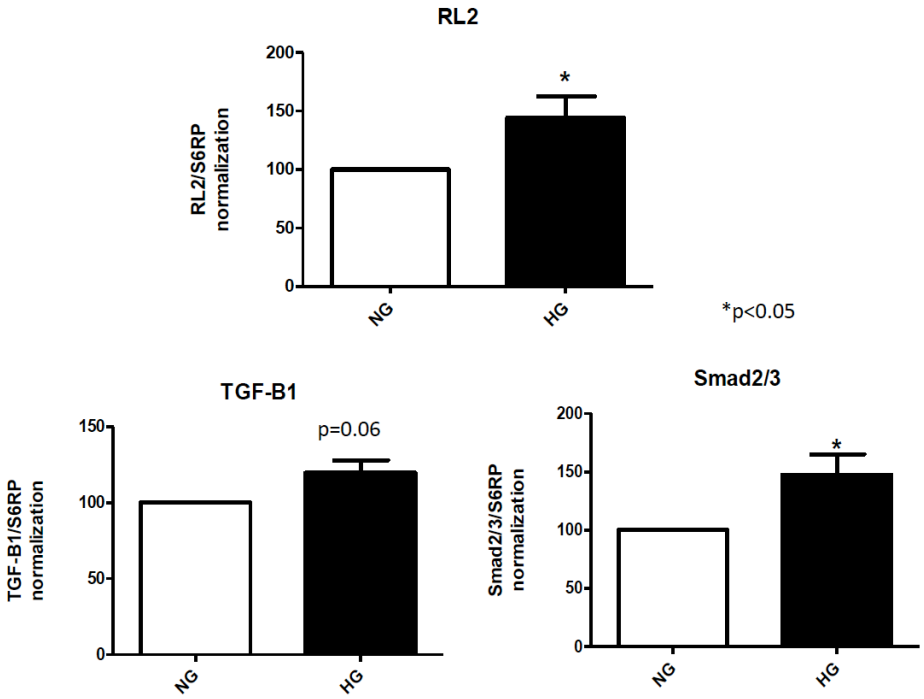

Figure 9. High glucose media effects on young, female (isolated from 3 month old) rat cardiac fibroblasts on total protein glycosylation (RL2 signal), TGF-β1 and Smad2/3 protein levels as per Western blots. Results from n=3 experiments each, in triplicate.

III. Opportunities for training

Although the project is not formally structured to provide training, we have now incorporated two PhD students into the project. The pre-doctoral fellows (Moises Bustamante and Elva Garate) are being trained in techniques related to in vivo physiology and pharmacology. Both students are fully committed to the project and we have trained them extensively on in vivo and ex vivo methods to assess for changes in cardiac structure and function and the impact that Epi treatment may yield on these endpoints. They are also involved in the implementation of cell culture studies using cardiac fibroblasts isolated from intact hearts. Training activities also include one-on-one mentoring with senior staff so as to achieve technical proficiency in the methods they are being exposed to. Moises Bustamante is the 1st author listed on the submitted manuscript and Elva Garate as a middle author. Both students are also listed as authors in the listed abstracts and had the opportunity to present the study results at the 2018 Experimental Biology meeting.

IV. Dissemination of results

We have disseminated our initial results during the Experimental Biology meeting held in San Diego in April of 2018. We also reported to the international heart failure community, the initial results of the effects of epicatechin in mitigating adverse changes driven by aging/estrogen and/or fructose induced metabolic syndrome at the European Society of Cardiology, Heart Failure meeting in May of 2018 in Vienna, Austria. We anticipate reporting on further advances to the local community (presentation scheduled for October 12th, 2018) and we will submit for presentation at National/International conferences in 2019.

V. Plans

The project has progressed well within the anticipated general plan as 2 (out of the 3) large in vivo studies have been completed generating a highly detailed database of results. As a clearer picture emerges from completion of the 3 animals studies we will be able to draw more solid conclusions as to the impact that each of the examined factors have on cardiac structure/function and on the capacity of Epi to limit/reverse these. All of the relevant data generated are going to be incorporated extensively into the proposed computer based simulations to examine the effect that changing specific variables may have on diastolic function in particular, if changes are hypothetically accentuated as per an even older phenotype than that studied so far. In vitro studies although progressing more slowly than anticipated, should quicken their pace so as to generate an ample body of mechanistic evidence for the antifibrotic effects of epicatechin.

IMPACT

We believe that the work being generated by this project is likely to unmask an important role for estrogens in mitigating the risk of developing adverse changes in cardiac structure/function that are slowly compounded with aging and that can remain undetected in female subjects for years. This area of cardiology is underappreciated, as there are a very limited number of publications that address these factors systematically. As the largest number of patients suffering from HFpEF are older women (~2/3 of the patient population), the loss of estrogen “protection”

may be recognized as an important contributor to the pathology in those at higher risk of developing the disease. As a side note, also 2/3 of the Alzheimer's population are women that largely present with similar risk factors (such as obesity and hypertension). If the loss of estrogen protection proves critical, then therapeutic strategies will need to be devised that reduce and/or control this risk factor in promoting the slow development of these age related pathologies. Furthermore, this study also lays the ground for the systematic analysis of differences in gender as it relates to the development of various types of cardiometabolic diseases.

CHANGES/PROBLEMS

As in year 1 of the project, only two challenges remain of significance and may or have required the following adjustments:

1. Aged female rats have an attrition rate of ~20% due to spontaneous death and development of cancerous tumor growth which requires euthanasia. As the project moves forward into its final year, we will eventually request for the approval for the use of a larger number of animals (over the original estimated total).
2. As noted above, to optimize the cell culture conditions of cardiac fibroblasts isolated from female hearts we are using media that is free of estrogens and phenol red.

No other matters have required of changes.

PRODUCTS

Journal Publications

1. Bustamante M, Garate-CarrilloA, Ito B, Garcia R, Carson N, Ceballos G, Ramirez-Sanchez I, Omens J, Villarreal F: Unmasking of Estrogen Dependent Left Ventricular Dysfunction in Aged Female Rats: A Potential Model of Early Stage HFpEF. Submitted, JACC Basic to Translational, 2018
[An outline of the submitted study is presented above].

2. Saucerman J, Tan P, Buchholz K, McCulloch A, Omens J. Mechanical regulation of gene expression in myocardium. Submitted, Nat Rev Cardiol, 2018
[Brief abstract: In this review, we summarize mechano-regulated pathways in cardiac cells that lead to altered gene expression and cell remodeling under physiologic and pathophysiologic conditions. Recent developments in systems modeling of the networks that regulate gene expression in response to mechanical stimuli should improve integrative understanding of their roles *in vivo* and help to discover new combinations of drugs and device therapies targeting mechanosignaling in heart disease].

3. Carruth E, The I, Schneider J, McCulloch A, Omens J, Frank L. Regional Variations in Diffusion Tensor Anisotropy are Associated with Myocyte Remodeling in Left Ventricular Pressure Overload. Submitted, Am J Phys, 2018
[Brief Abstract: In this study we hypothesized that regional (especially transmural) gradients in structural properties of myocardium would become more spatially uniform and would be reduced

in pressure overload such as seen with hypertension to maintain uniformity of fiber strain. To test our hypothesis, we performed high-resolution, high-fidelity Diffusion Tensor-MRI (DTI) on rat hearts isolated/fixed from transverse aortic constricted and sham control animals and investigated the regional variations in DTI-derived parameters of orientation, diffusivity, and anisotropy in the LV. We found that there are indeed regional variations in myocyte geometry and structural organization, which become more uniform with pressure overload. Additionally, several structural features correlated significantly with DTI-derived parameters, regardless of phenotype. These results will be valuable in understanding the mechanisms by which cardiac myocytes in different regions of the LV respond to hypertension and may provide a tool for diagnosing the early stages of hypertrophy or other remodeling clinically and non-invasively, allowing for earlier lifestyle changes or interventions to reverse early stage remodeling before the progression to heart failure occurs].

Conference Papers

1. Bustamante M, Garate-Carrillo A, Loredo M, Garcia R, Carson N, Ito B, Ceballos G, Omens J, Ramirez-Sanchez I, Villarreal F. Detrimental Effects of Aging, Ovariectomy and Weight Gain on Left Ventricular Structure and Function: A Potential Preclinical Model of Early Stage HFpEF. FASEB J, 32, S1, 2018

2. Bustamante M, Garate-Carrillo A, Ito B, Ceballos G, Omens J, Ramirez-Sanchez I, Villarreal F. Development of an aging female rat model of HFpEF and evaluation of the antifibrotic potential of (-)-epicatechin. European Journal of Heart Failure, 20, S1, 2018

Presentations

1. Presented Conference Paper listed as #1 above at the Experimental Biology meeting in April, 2018 in San Diego, CA

2. Presented Conference Paper listed as #2 above at the European Society of Cardiology, Heart Failure meeting in May, 2018 in Vienna, Austria

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

I. Individuals involved in the project

Francisco Villarreal (Principal Investigator) no change
Jeffrey Omens (Co-Investigator) no change
Israel Ramirez-Sanchez (Project Scientist) no change
Diane Huang (SRA 2) no change

Name	Moises Bustamante
Project role:	Graduate student
ID	114959
Cal months	12
Contribution to project	In vivo physiology and pharmacology
Funding support	CONACyT and DoD (this award)

Name	Elva Garate
Project role:	Graduate student
ID	000921750
Cal months	12
Contribution to project	In vitro biology and in vivo pharmacology
Funding support	CONACyT and DoD (this award)

II. Changes in other support

Nothing to report.

III. What other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Not applicable

APPENDICES