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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

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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.

ACCOMPLISHMENTS

I. Major goals of the project

Major Goals: The following are the major tasks identified in the State 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 (75% completed)
2. Characterize effects of early Epi treatment on myocardial fibrosis in a model of estrogen depletion and aging (10% completed)
3. Characterize effects of early Epi treatment on myocardial fibrosis in a model of estrogen depletion, aging and fructose supplementation (10% completed)

Aim 2 related: Late treatment with Epi reverses myocardial fibrosis

4. Characterize long-term baseline effects of aging on myocardial fibrosis (75% completed)
5. Characterize the reversal of myocardial fibrosis by late Epi treatment in a model of estrogen depletion and aging (10% completed)
6. Characterize effects of estrogen depletion, aging and fructose supplementation on myocardial fibrosis and its reversal by late Epi treatment (10% completed)

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

7. Cardiac fibroblast phenotype characterization (10% completed)
8. Effects of profibrotic phenotype stimulation/inhibition (pending)
9. Gene expression modulation (pending)

II. Accomplishments

A major part of the project's major endeavor during this initial period of support relates to the implementation of the female model of aging driven cardiac remodeling and fibrosis that can be compounded by ovariectomy and fructose supplementation (as stated above in goals #1 and 4). Obtaining aged female rats from the established NIH/NIA colony initially required 1.5 months of quarantine and to allow for their further aging as per the approved research plan and protocol. An unanticipated occurrence was the ~10% "spontaneous" death rate due to aging and an additional ~10% incidence of aging associated cancerous tumor growth that requires early euthanasia. Thus, the current attrition rate is ~20%. We have begun to extensively characterize changes in cardiac structure/function that develop as a function of aging (alone), aging + ovariectomy and aging + ovariectomy + fructose supplementation. As stipulated in the project plans we have pursued the following:

- Assessed serial changes in cardiac structure/function using echocardiography
- Implemented terminal studies and recorded detailed 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 epicardial LV strains
- Fixed hearts and recorded histomorphometric parameters

Methods utilized include standard echocardiography to measure in vivo changes in gross heart morphology, diastolic and systolic function. In vivo LV hemodynamics utilized the Millar pressure conductance catheter. At the time of the terminal study, an ex vivo assessment of LV pressure volume and strain is implemented using an inflatable balloon as well as video recording of the inflating hearts. Finally, hearts are then fixed and sectioned for detailed histological analysis using H&E and trichrome staining.

The results gathered so far have been summarized in abstract form and have been submitted for consideration for presentation at the 2017 American Heart Association meeting to be held in Anaheim in the month of November. The abstract text reads as follows:

Adverse Effects Of Aging, Estrogen Depletion And Fructose Supplementation On Left Ventricular Structure And Diastolic Function: An Aging Female Rat Model For Early HFpEF
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 would assist in novel therapy evaluation. We wished to develop a female animal model of aging, estrogen depletion and metabolic syndrome to evaluate the role of these factors in altering cardiac structure/function. One and half year old female Fischer F344 rats were allocated into an aging group, aging + ovariectomy and aging + ovariectomy + 10% fructose in drinking water (n=8-10/group). At 22 months of age, animals were anesthetized and left ventricular (LV) function was evaluated using a Millar conductance catheter. Histological measures of chamber morphometry, inflammation and collagen density were also obtained. Intraventricular PV loop analysis (see figure) evidenced significant decreases in stroke work (SW; 33-35%), cardiac output (CO; 40-43%) and increases

in end-diastolic pressure volume relationships (i.e. stiffness) with ovariectomy (EDPVR; 130%). Histomorphometry did not identify changes in LV/body weight. However, histological analysis indicated progressively increasing levels of inflammatory infiltration, perivascular and interstitial fibrosis with ovariectomy and with fructose supplementation. In conclusion, in the setting of aging, ovariectomy (which mainly leads to estrogen deprivation), markedly deteriorates myocardial microstructure which may facilitate the loss of diastolic and systolic function. This model may serve to better understand the role that aging and menopause may have in the development of HFpEF [**end of abstract**].

Interestingly, the results gathered so far, have documented no major development of cardiac hypertrophy after tracking animals serially using echocardiography over a period of 3 months. These data were confirmed using post-mortem morphometrics (heart and LV weights normalized to body weight). Also preliminary assessment of echocardiographic indices of systolic function during their evolution evidence no major apparent alterations. Currently, we are analyzing data on diastolic parameters so as to establish the impact of interventions on these endpoints. In contrast, at the terminal study, the analysis of intraventricular (i.e. LV) hemodynamics has evidenced significant changes in both diastolic and systolic indices of function. The figure shown below illustrates the parameters that have reached statistical significance so far (n=6/group, *p<0.05 by ANOVA). A sensitive index of myocardial compliance (i.e. stiffness) is the end-diastolic pressure volume relationship (EDPVR). Results indicate that as compared to aging only rats, ovariectomy (OVX) significantly increases the calculated slope for the EDPVR suggesting higher levels of LV stiffness. Stroke work (SW) and cardiac output (CO) values recorded also indicate significant losses in systolic function with ovariectomy alone and in those supplemented with fructose that in our experience are equivalent to those that occur in rats subjected to ischemia/reperfusion injury and are evaluated using the same system 4 weeks after infarction.

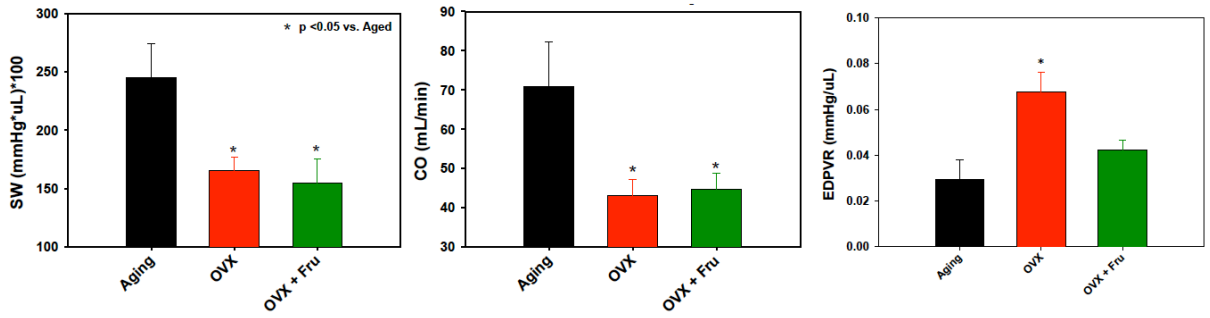

Figure 1. Changes in stroke work (SW), cardiac output (CO) and end-diastolic pressure volume relationship (EDPVR) in aged rats undergoing ovariectomy (OVX) alone or in combination with fructose (Fru) supplementation.

The analysis of LV pressure-volume curves and epicardial strain data is currently being pursued and results should be compiled within the next few weeks. The preliminary analysis of histological images (example shown below from an animal with ovariectomy and fructose) has evidenced high degrees of an inflammatory infiltrate, interstitial and perivascular fibrosis, which may underlie the changes in diastolic and systolic function, noted above.

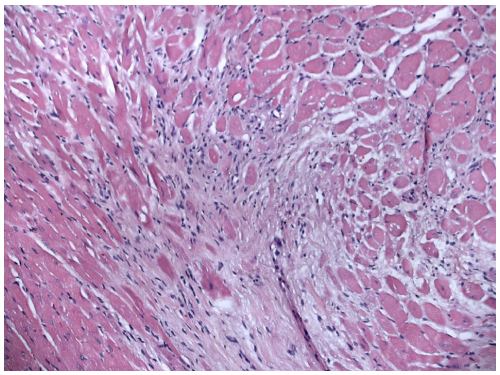

Figure 2. Histological section from the LV free wall in a 22 month old female rat subjected to ovariectomy and fructose in water (20 X magnification).

As the full characterization of the untreated groups is currently underway, additional animals are being allocated to each of the subgroups. A more extensive analysis of data should yield greater validation of the preliminary changes noted and reached greater levels of statistical significance. Pertaining to the groups of animals to undergo Epi exposure (early and late), these are currently at their very early stage of treatment and we anticipate to have accomplished a preliminary evaluation of the effects during the course of year 2 of the award.

As it relates to aim 3 studies, using cultured cardiac fibroblasts we have begun to implement the culture system and are in the process to begin to assess the effects of interventions such as high glucose treatments.

III. Opportunities for training

Although the project is not formally structured to provide training, we have managed to incorporate a graduate student into the project who currently holds a Masters degree and is pursuing his PhD studies. The pre-doctoral fellow (Moises Bustamante) is being trained in techniques related to in vivo pharmacology. He is fully vested in this project and we have exposed him to in vivo and ex vivo methods to assess for changes in cardiac structure and function and the impact that Epi treatment will yield on these endpoints. Training activities also include one-on-one mentoring with senior staff so as to achieve in the near future, technical proficiency in the methods he is being exposed to. The student is also noted as a co-author in the submitted AHA abstract and is expected to attend the National meeting in November.

IV. Dissemination of results

As stated above, we have submitted an abstract for consideration to the National AHA 2017 meeting. A notification on the possible acceptance will occur in September. We have opted to provide either an oral or poster presentation. We also anticipate to disseminate our initial results

in extenso during the Experimental Biology meeting to be held in San Diego in 2018. The due date for such abstracts is November 2017.

V. Plans

The project is in the initial phase where an extensive characterization of the effects that aging, ovariectomy and fructose supplementation yield on cardiac structure/function with an emphasis on fibrosis and changes in tissue compliance. We expect that by the next reporting period, a full submission of a research article would have occurred on this subject. We also expect that the 2nd phase related to the effect of Epi treatment would have completed its preliminary stage. Studies on the effects of treatments on cultured cardiac fibroblasts would have also completed their initial phases.

IMPACT

As the project is in year 1 of implementation it is premature to denote verifiable accomplishments. However, the preliminary nature of the changes noted and their apparent "severity" make us predict that the loss of estrogen (albeit at the low levels that occur with aging) appear to contribute greatly to the relative preservation of cardiac health and could potentially be recognized as a major driver of the pathophysiology of HFpEF patients.

CHANGES/PROBLEMS

Only two challenges have emerged during this period.

1. As noted above, the aged female rats have an attrition rate of ~20% due to spontaneous death and development of cancerous tumor growth which can require euthanasia. As the project moves forward we may require to make changes in the vertebrate animal protocol to require approvals for the use of a larger number of animals.
2. Apparently, the culturing of female rat cardiac fibroblasts is more challenging than their male counterpart cells. This may be due to the presence or absence in serum (used to culture cells) of estrogens or other factors. We are currently adjusting culture conditions to account for this apparent limitation.

No other matters have required of changes.

PRODUCTS

Nothing to report.

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)

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

Nothing to report.