AWARD NUMBER: W81XWH-17-1-0507

TITLE: Gender and Gulf War Illness

PRINCIPAL INVESTIGATOR: Mariana Morris, PhD

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REPORT DATE: DECEMBER 2020

TYPE OF REPORT: Final Technical Report

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**
DECEMBER 2020	FINAL	15 Aug 2017 - 14 Aug 2020

4. TITLE AND SUBTITLE

Gender and Gulf War Illness

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0507

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Nova Southeastern University, Inc.
Fort Lauderdale, FL 33314

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

Gulf War Illness (GWI) is a persistent disorder characterized by dysfunction of the immune, neuroendocrine and autonomic nervous systems. Although both genders were deployed to the military theater, most studies of GWI have focused on males. We used the sarin surrogate diisopropyl fluorophosphates (DFP) coupled with treatment with the adrenal hormone, corticosterone (CORT) to develop an animal model of GWI, characterized by neuroinflammation and heart disease. We propose a program to extend our GWI animal model to female. The goal is to characterize the inflammatory, endocrine and autonomic/ cardiac characteristics. The need for such a program is based on the lack of detailed information on the etiology of GWI in females. This knowledge will allow us to proceed down the path to feasible GWI treatments. Objective: The objective of this proposal is to use a GWI female mouse model to study cardiovascular and neuro-inflammatory profiles to DFP exposure. We suggest cardiac/autonomic dysfunction caused by neuroinflammatory and cardiovascular response to organophosphate toxins due to the interactions between the gender specific sex steroids, the extended neuroendocrine and immune systems, and the adrenal stress axis

15. SUBJECT TERMS-

NONE LISTED

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
U	U	U	UU	21	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Gulf War Illness (GWI) is a long-lasting, multi symptom disorder with features characteristic of "sickness" behavior 1-3. Symptoms include cognitive impairment, fatigue, depression, sleep disruption, muscle/joint pain, and autonomic dysfunction 4-7 . Typically, sickness behavior resolves with time 8; however, in GWI symptoms persist, suggestive of heightened neuro-inflammation. There are important questions as to how females respond to GWI. To date, most GWI studies have been conducted in males although, both genders were deployed to the Persian Gulf. Furthermore, there is an increasing representation of females in the U.S.A military. The aim of this proposal is to use a GWI female mouse model to study cardiovascular and neuro-inflammatory profiles as well as the influence of therapeutic targets for the immune and adrenal systems (specifically Tumor Necrosis Factor alpha (TNF-α) and adrenocorticoid receptors). Drug treatments will include Enbrel (ENB) to target TNF-α and Mifepristone (MIF) to target adrenocorticoid receptors. The project is appropriate for Tier 1 status based on discovery of new experimental and clinically relevant results. Innovation is an important aspect of the program as related to GWI model development and testing. The hypothesis is related to the idea that the disease process is different between genders and that any information on treatment will require complete integrative data sets.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Autonomic Dysfunction, Cytokines, Deregulated Balance, Diisopropyl Phosphorofluoridate , Electrocardiogram, Gulf War Illness, Stress Response, Target Intervention, Therapeutic Interventions.

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Major Task 1: Training and educational development	Timeline (Months)	Percentage Complete
Subtask 1: Set up programmatic structure, hire and train personnel	1-4	100%
Subtask 2: Review and refine research protocols	1-4	100%
Subtask 3: Submit requests for organophosphate use from biohazard committee	1-6	100%
Subtask 4: Submit animal use documents for approval to IACUC	1-6	100%

Milestone Achieved: Research staff trained, platform tested	6	100%
Milestone Achieved: Local IACUC, Biohazard approval at NSU	6	100%
Milestone Achieved: Study begins	6	100%
Major Task 2: Conduct studies of cardiovascular, immunological and endocrinological effects of DFP		
Subtask 1: Conduct preliminary experiments for testing effects of DFP	2-6	100%
Subtask 2: Begin long term study with the effect of DFP on immunological, cardiovascular and endocrine parameters in female OVX	6-11	100%
Subtask 3: Finish experiments in DFP treated female OVX	12	100%

What was accomplished under these goals?

Dr. Morris group presented following posters:
1. Jacqueline F. Machi, Luis M. Salgueiro, Rodrigo Schmidt, Nancy Klimas, Mariana Morris. PRE-clinical animal model: Drug treatments are a success to reduce cardiovascular toxicity to DFP exposure. 2017 ESRF 43rd Annual Eastern-Atlantic Student Research Forum (February 2225, in Miami FL) 2017.
2. Jacqueline F. Machi, Luis M. Salgueiro, Rodrigo Schmidt, Nancy Klimas, Mariana Morris. Cardiac Function in a Murine Model of Gulf War Illness (GWI): Success in Therapeutic Trial. 2017 SOT 56th Annual Meeting and Tox Expo (March 12-16, in Baltimore, Maryland) 2017.
3. Rodrigo Schmidt, Luis M. Salgueiro, Jacqueline F. Machi, Nancy Klimas, Mariana Morris. Organophosphate (OP) Toxicity Model in Female Mice – Heart Rate Variability to Access the Autonomic Balance. 2017 SOT 56th Annual Meeting and Tox Expo (March 12-16, in Baltimore, Maryland) 2017
4. Influence of therapeutic target and cardiovascular profile: A Female Neurotoxin Model. Rodrigo Schmidt, Luis M. Salgueiro, Jacqueline F. Machi, Nancy Klimas, Mariana Morris. Glucocorticoid Antagonist (Mifepristone) Treatment: Effects on Autonomic Imbalance in a Murine Model of Gulf War Illness. 2017 SOT 56th Annual Meeting and Tox Expo (March 12-16, in Baltimore, Maryland) 2017.

What opportunities for training and professional development has the project provided?

Dr. Morris had 4 new students that joined the lab during the execution of these projects. They worked together on collaborating with some analysis such as Histology and Heart Rate Variability for the Gender and Gulf War Illness*. There were also continuing opportunities for training.*

How were the results disseminated to communities of interest?

The key part of this research program is the dissemination of information to a wider audience. This means that critical information will be available to the scientific community. The dissemination to a wider audience will happen by presentations at international, national and local meetings.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Critical methods were developed and experiments were completed. This has significant impact on the total project.
The project has stablished gender differences on GWI symptoms management. The development of this project produced knowledge that allowed the PI to be awarded with two NSU President's Faculty Development and Research Awards:
1 - 2017 - $15,000 to develop the project entitled "Dietary fructose as a Risk factor for Gulf War Illness: Exercise Challenge". Nova Southeastern University.
2 - 2018 - $15,000 to develop the project entitled "Neurotoxin Model: Cardiovascular, Immune and Oxidative Stress Profile". Nova Southeastern University

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

No major changes to report

Actual or anticipated problems or delays and actions or plans to resolve them

No problems to report

Changes that had a significant impact on expenditures

No changes to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals.

No major changes to report

Significant changes in use of biohazards and/or select agents

No major changes to report

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

Publications are in progress. "Female Mouse Model of Gulf War Illness: the effects of etanercept and mifepristone on cardiovascular and autonomic outcomes" is being submitted.

  Books or other non-periodical, one-time publications

Not applicable

  Other publications, conference papers, and presentations.

Not applicable.

Not applicable.

- **Technologies or techniques**

Not applicable.

No applicable.

- **Other Products**

Not applicable.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Mariana Morris, PhD
Project Role:	PI
Research Identifier:	eCommons: mariana
Nearest person month worked:	4.8
Contribution to Project:	Overseeing the entire research project. Established the animal protocols and in charge of the animal research. Oversees hiring all personnel.
Funding Support:	NIH

Name:	Jacqueline Machi, PhD
Project Role:	Research Associate
Research Identifier:	None
Nearest person month worked:	12
Contribution to Project:	Active in animal experiments
Funding Support:	None

Name:	Rodrigo Schmidt, PhD. MS.
Project Role:	Research Associate
Research Identifier:	None
Nearest person month worked:	12
Contribution to Project:	Active in animal experiments
Funding Support:	None

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Task: DFP and drugs therapies –Morphology of the heart, ECGenie and Echocardiography analysis.

The third phase of the male and female studies were to evaluate the effects of DPF exposure on heart, for this, morphometric parameters were assessed in H&E stain. Briefly, the hearts were fixed in 10% phosphate-buffered formalin solution and they were serially sectioned in 2-mm-thick cuts from base to apex following Cavalieri's method[1] (Fig A). The samples were dehydrated in graded alcohol series, embedded in paraffin wax and serially sectioned using a microtome. Random serial sections of 5 µm thickness of each section were mounted onto glass slides and H&E stain was performed. These slides were scanned and the morphometric analyses were done by NIH ImageJ software. The left ventricle wall, right ventricle wall, lumen and wall thickness of the left ventricle as well the left ventricle area were measured.

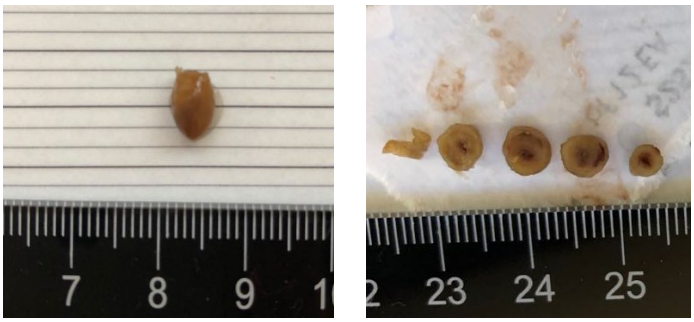
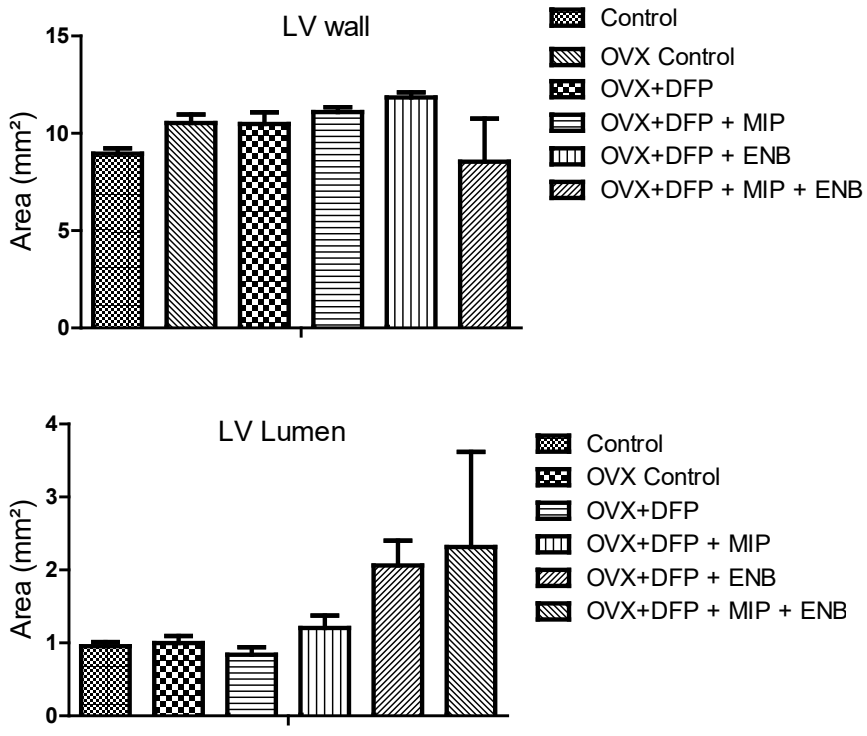


Figure **A**. Heart sampling. Transversal cuts with 2 mm thickness.

Reference
1- Gundersen HJ, Bendtsen TF, Korbo L, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 1988; 96: 379–394.

We did not observe differences among groups for LV wall and LV lumen areas in both Female studies.

Female Protocol Morphology



Parameter	1 - Control	2 – OVX Control	3 – OVX DFP	4 - ENBREL	5 - MIF	6 - COMB	p-*Value*
LV Wall (mm)	0.95±0.068	0.92±0.073	1.15±0.062	0.98±0.053	1.08±0.050	1.08±0.044	0.0747
RV Wall (mm)	0.43±0.053	0.35±0.035	0.38±0.032	0.38±0.019	0.40±0.029	0.40±0.036	0.7667
LV Lumen Area (mm^2)	0.95±0.058	0.99±0.095	0.83±0.102	2.06±0.339	1.20±0.168	2.31±1.30	0.4567
LV Wall Area (mm^2)	8.95±0.27	10.52±0.44	10.48±0.58	11.84±0.26	11.09±0.24	8.53±2.21	0.2588

Figure B - Data obtained from heart morphometric analyses. LV wall diameter (mm), RV wall diameter (mm), LV lumen area and LV wall area. Data are presented as mean ± standard error of the mean. Data were analyzed using ANOVA and Bonferroni post hoc test (n=10). 1– Female Control; 2- Female OVX Control; 3 – OVX GWI Model (OVX+Cort+DFP); 4– OVX GWI Model + MIF; 5– OVX GWI Model + Enbrel; 6– OVX GWI Model + Enbrel + MIF.

Female Protocol - ECGenie

Morris group is using one new methodology: The ECGenie is a non-invasive electrocardiogram (ECG) recording system that is used in conscious mice. This method of recording ECGs uses a lead II ECG attached to the paws. It records the P-R, QRS, QT intervals and HR. ECG analyses software, provided by the company, Mouse Specifics, analyzes the signals to assess animal health, cardiac diseases, and drug toxicity.

Parameter	1 - Control	2 – OVX Control	3 – OVX DFP	4 - ENBREL	5 - MIF	6 - COMB	p-*Value*
RR (ms)	79.75±1.20	78.96±0.57	79.36±0.51	80.75±0.63	80.54±0.38	79.35±0.44	0.2686
PR (ms)	28.1±0.583	26.50±0.717	27.52±0.467	29.08±0.558	27.89±0.837	26.38±0.831	0.0726
QRS (ms)	10.07±0.447	10.55±0.138	10.83±0.124	10.71±0.184	10.64±0.168	10.50±0.278	0.2795
QT (ms)	39.58±0.805	40.20±0.582	39.84±0.835	40.81±0.448	40.23±0.805	40.87±0.735	0.8284
ST (ms)	28.15±1.66	30.01±0.546	29.50±0.842	30.60±0.540	30.09±0.751	30.88±0.737	0.3949

Figure C – ECG segments obtained after drugs treatment. ECG intervals are presented as mean ± standard error of the mean. Data were analyzed using ANOVA and Bonferroni post hoc test (n=10). 1– Female Control; 2- Female OVX Control; 3 – OVX GWI Model (OVX+Cort+DFP); 4– OVX GWI Model + MIF; 5– OVX GWI Model + Enbrel; 6– OVX GWI Model + Enbrel + MIF.

Female Protocol <u>Echocardiography (echo)</u>

Echo was performed to assess cardiac structure and function before (baseline), after DFP exposure, and after therapy (treatment with Enbrel, Mifepristone and combined). The low dose of DFP exposure produced increase on end systolic area (ESA) (**Figure D)**. The treatment with Enbrel, mifepristone or the combination produced decrease in ESA ameliorating the toxic change produced by DFP. End diastolic area (EDA) was not altered in DFP exposed mice. **(Figure E).**

Systolic function evaluated by ejection fraction **(Figure F)** was reduced in all DFP groups after exposure and remained in the last evaluation in DFP group when compared to control. Differences that were not observed in Enbrel, Mifepristone or combination group in relation to the control and OVX groups in the last evaluation. Fraction Shortening (FS) was reduced after DFP exposure in all groups (**Figure G**) when compared to control, OVX and their baselines. Final echocardiographic evaluation (after drug intervention) shows enhanced shortening, revealing the efficacy of the drug. **Figure H** shows that cardiac output (CO) was decreased after DFP exposure in all groups compared to control and OVX and their baseline. After drug treatment CO was increased in Enbrel, Mifepristone and combination when compared to DFP group, suggesting recovery of function.

Myocardial Performance Index (MPI) was obtained from Doppler for mitral flow and calculated using ejection time, isovolumetric contraction and relaxation time. MPI which is the ratio of total activity (index of cardiac overload) was significantly increased in DFP exposure mice and re-established in all groups that received therapeutic trial. DFP group was the only one that didn't re-establish their baseline values (**Figure I**).

Figure D

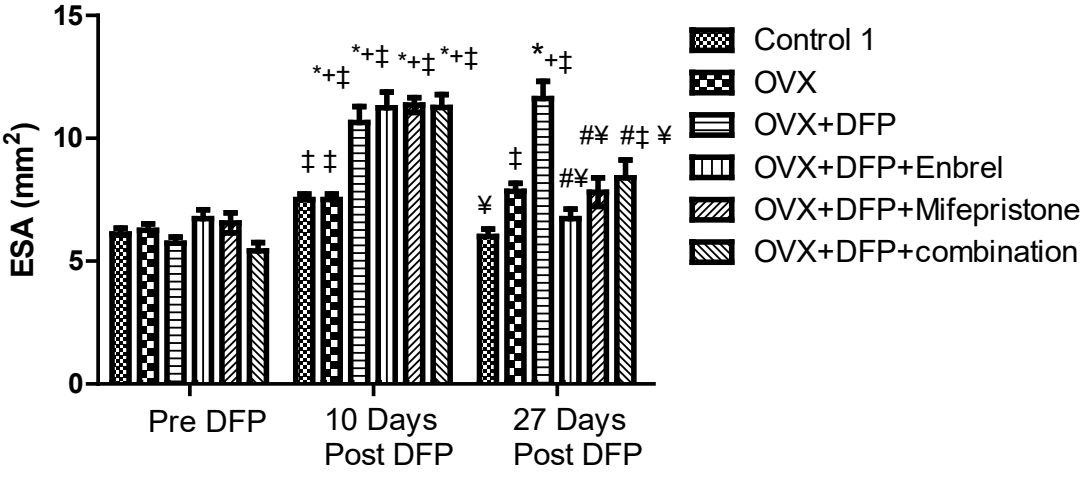


Figure E

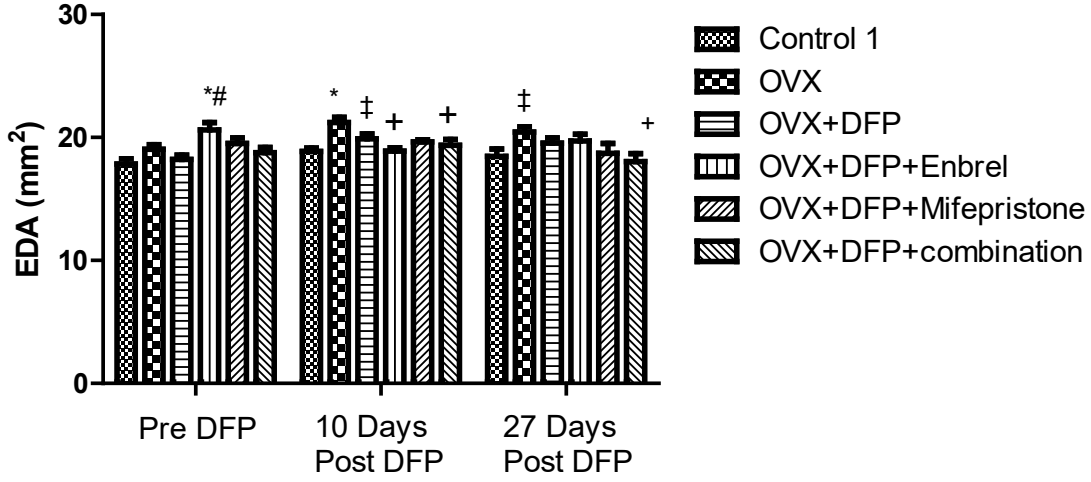


Figure F

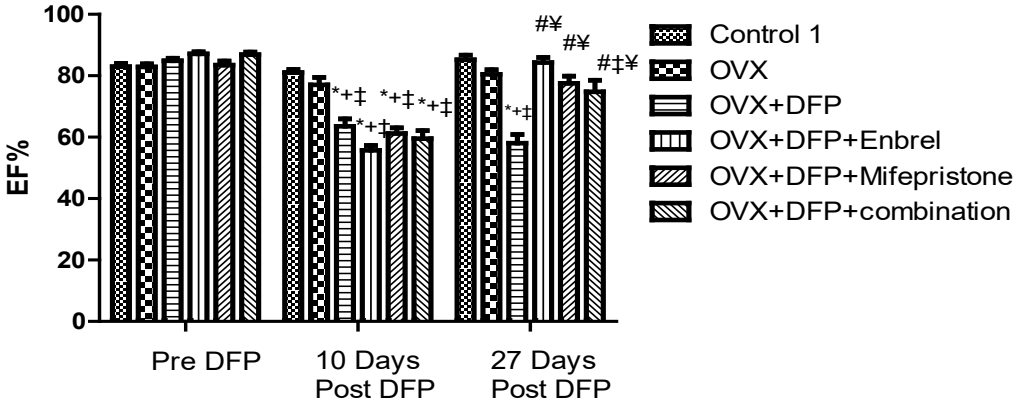


Figure G

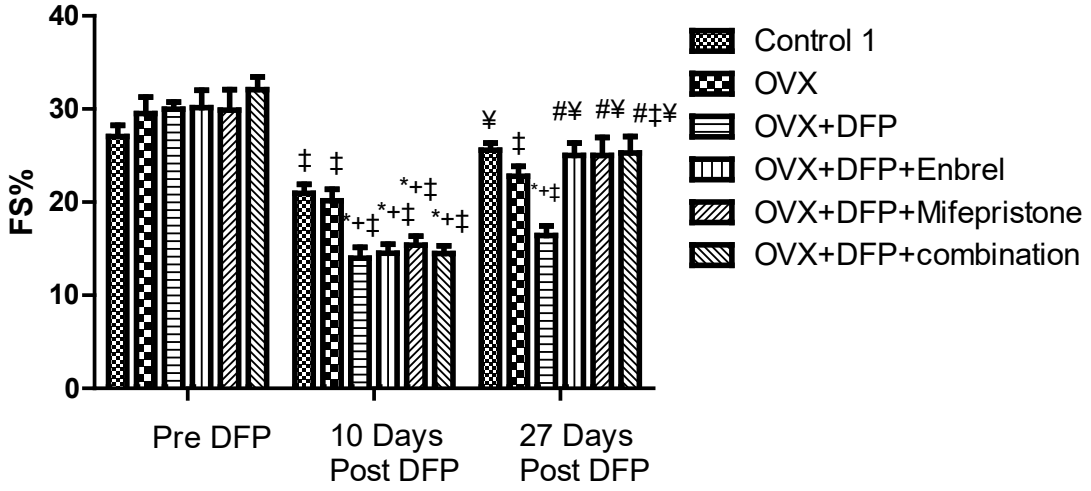


Figure H

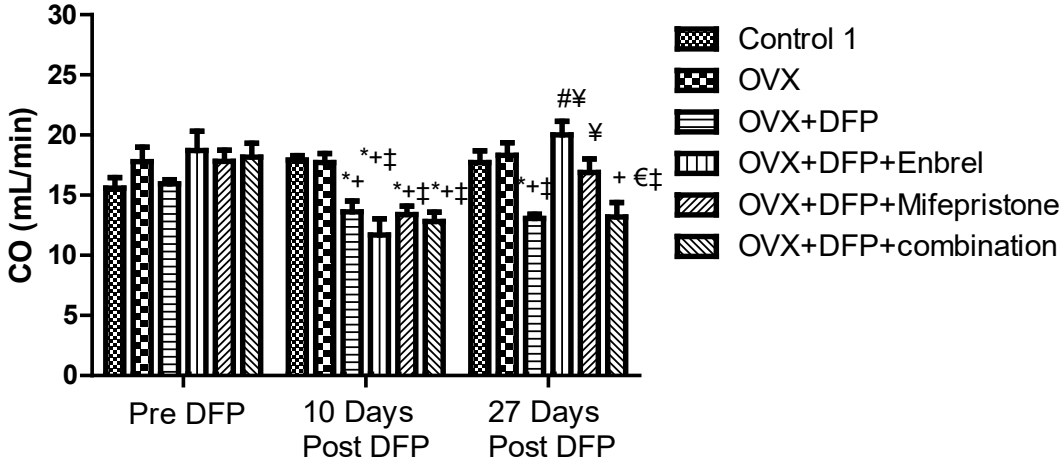
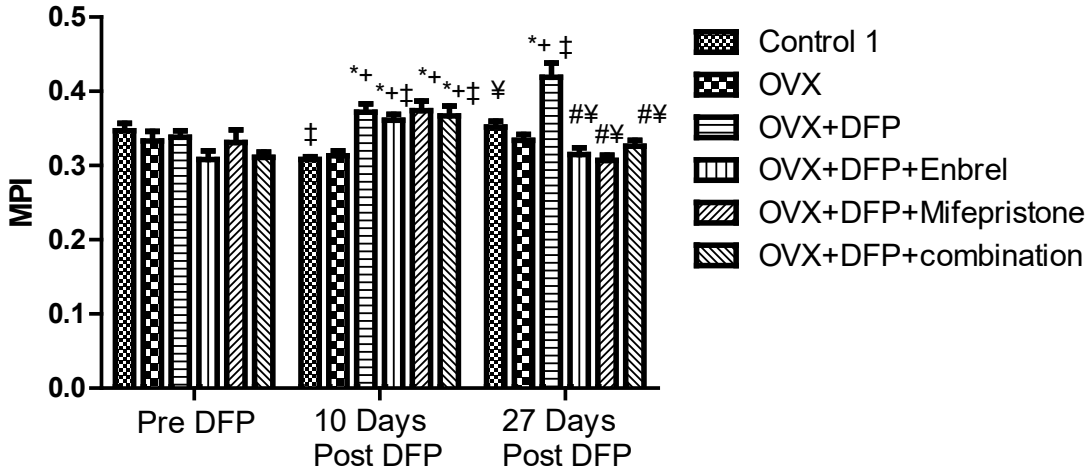


Figure I



Figures – D- End systolic area (ESA), **E-** End Diastolic area (EDA), **F-** Ejection Fraction (EF %), **G-** Fraction Shortening (FS %), **H-** Cardiac Output (CO), **I-** Myocardial Performance Index (MPI). Data are represented as mean ± standard error of the mean $p < 0.05$. * Versus control, + versus OVX, # versus DFP, € versus DFP+ Enbrel, † versus DFP + Mifepristone, ‡ versus initial of the same group, ¥ versus intermediate of the same group. Data were analyzed using ANOVA and Bonferroni post hoc test (n=8).

ECHO Male vs Female

Figure J - Ejection Fraction EF (%) Male vs Female

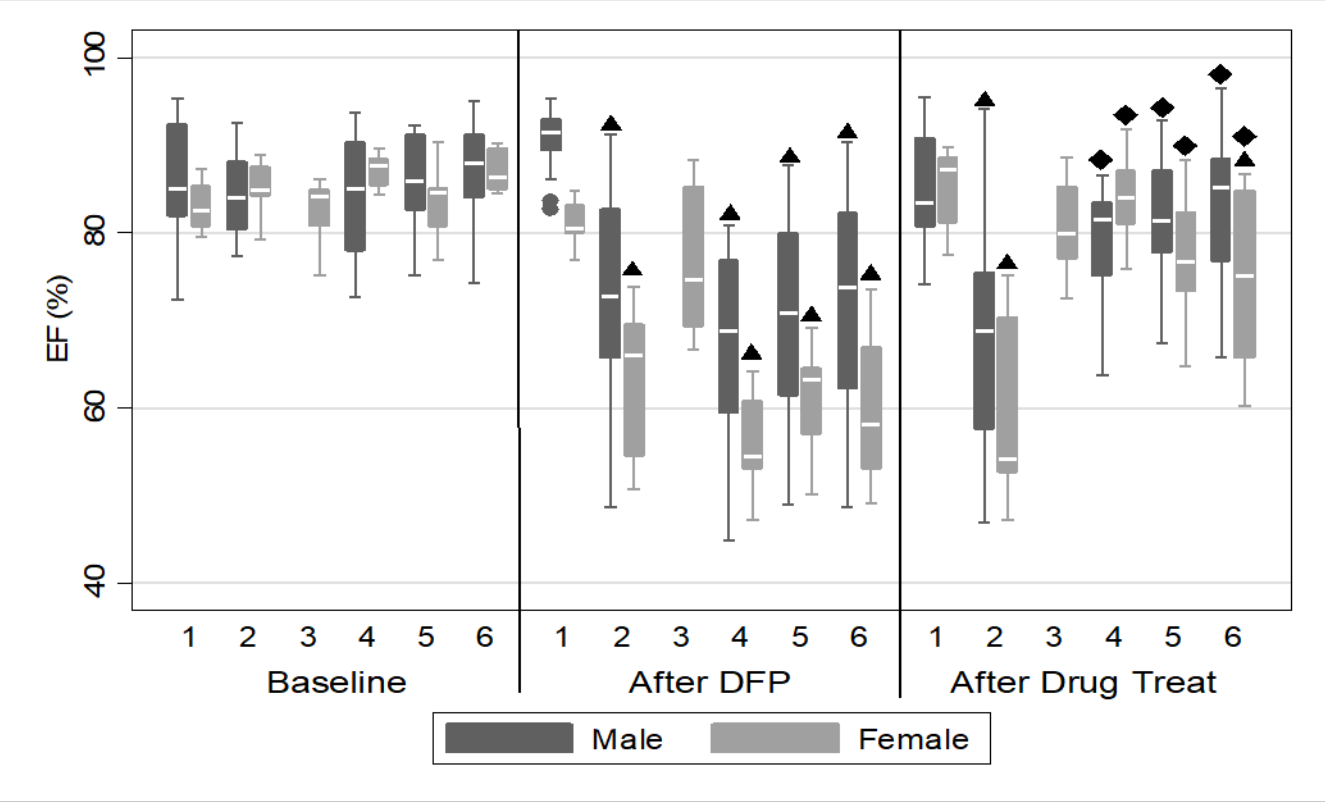


Figure J: Ejection Fraction (EF (%)), ▲=p≤0.05 versus baseline of the same group, ♦=p≤0.05 versus "After DFP" of the same group. Data were analyzed using ANOVA and Bonferroni post hoc test (n=10).
1 – Control Group (Male and Female Intact)
2 – GWI Model – CORT+DFP (Male and Female OVX)
3 – Control OVX (Female)
4 – GWI Model + Enbrel (Male and Female OVX)
5 – GWI Model + MIF (Male and Female OVX)
6 – GWI Model + Enbrel + MIF (Male and Female OVX

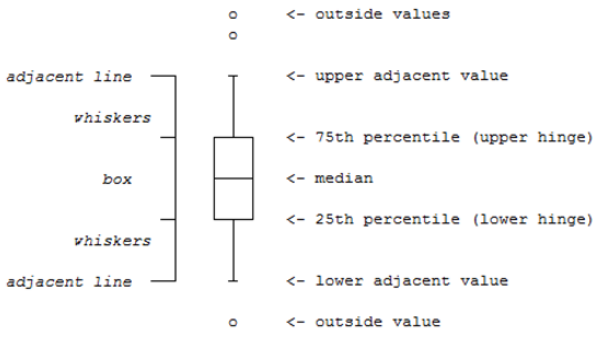


Figure K - End Systolic Area ($mm^{2)}$ Male vs Female

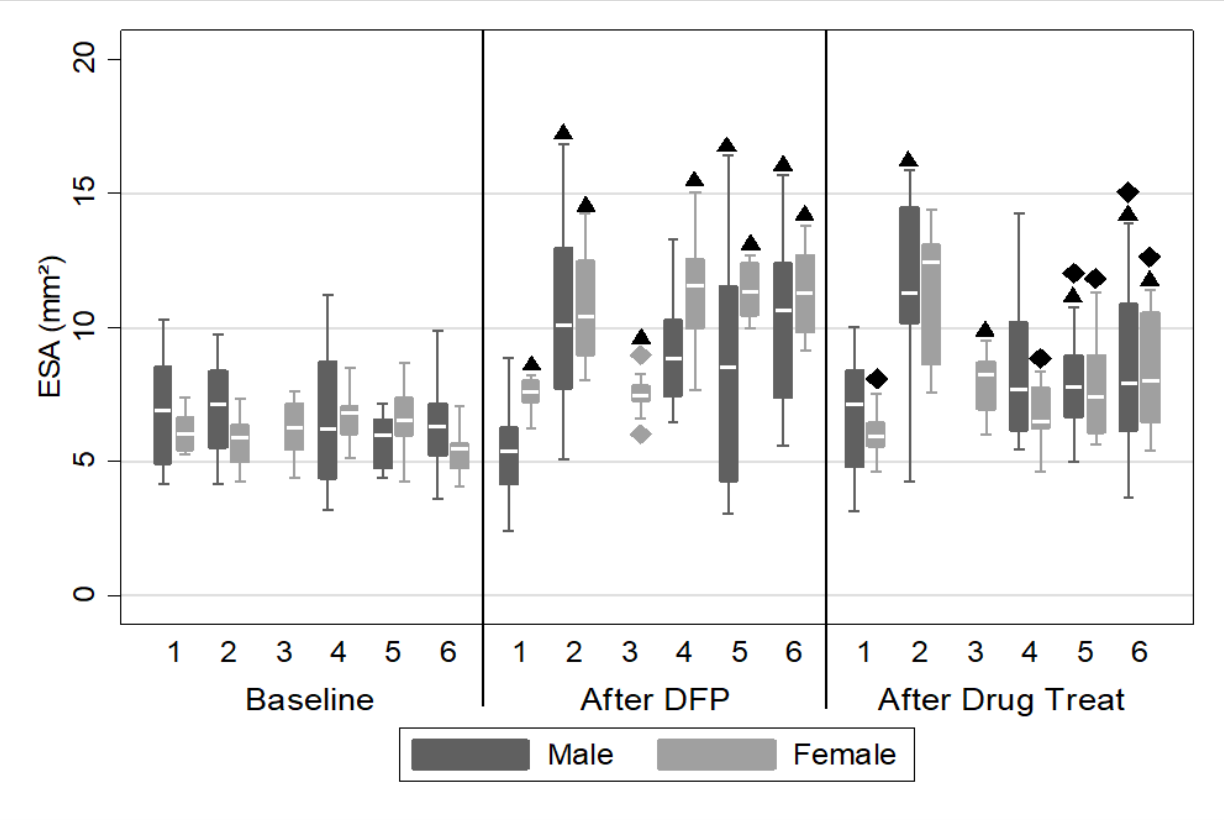
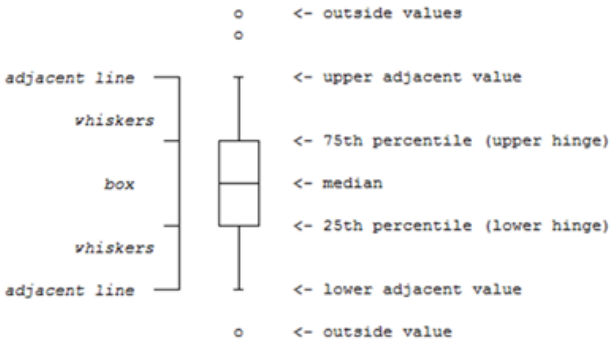


Figure **K**: End Systolic Area (ESA (mm^2)), ▲=p≤0.05 versus baseline of the same group, ♦=p≤0.05 versus "After DFP" of the same group. Data were analyzed using ANOVA and Bonferroni post hoc test (n=10).
1 – Control Group (Male and Female Intact)
2 – GWI Model – CORT+DFP (Male and Female OVX)
3 – Control OVX (Female)
4 – GWI Model + Enbrel (Male and Female OVX)
5 – GWI Model + MIF (Male and Female OVX)
6 – GWI Model + Enbrel + MIF (Male and Female



Autonomic Modulation accessed by Heart Rate Variability

The ECG wave was collected for a 3 min period using Vevo 1100 ultrasound system (FUJIFILM, Visual Sonics Inc., Toronto, Ontario, Canada). During this procedure, mice were anesthetized (Isoflurane, 1.5 – 2% on 0.8l/min of O_2) and attached to a board with ECG sensors. Data were exported as a *csv* format file for analysis using Windaq® Software (DATAQ Instruments, Akron, OH, US). We used one algorithm to detect R peaks and create a time series file containing all R to R intervals (RRI). A visual inspection was made one by one to determine Normal to Normal intervals (NNI) to build the tachogram without artefacts and perform the HRV analyses. Three spectral components were included: very low frequency (VLF), from 0 to 0.10 Hz; low frequency (LF), from 0.10 to 1.00 Hz; and high frequency (HF), from 1.00 to 5.00 Hz (Rodrigues, F.L., et.al., 2011).

Figures L, M, N present preliminary data regarding autonomic modulation in female mice

Figure L – Sympathetic Modulation LF (nu)

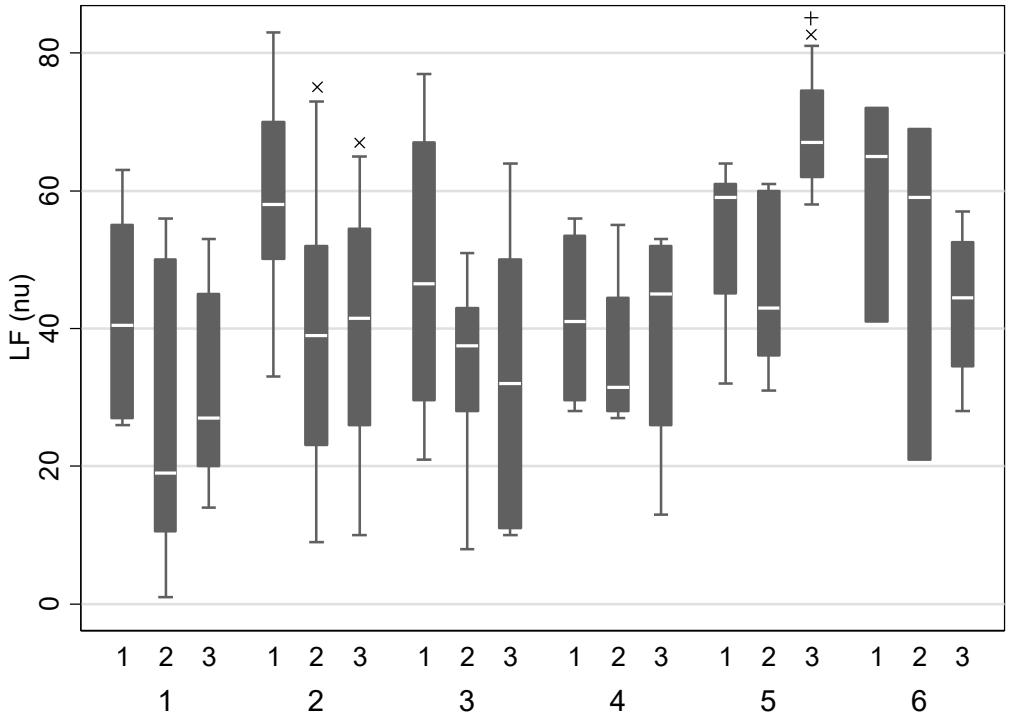


Figure M – Parasympathetic Modulation HF (nu)

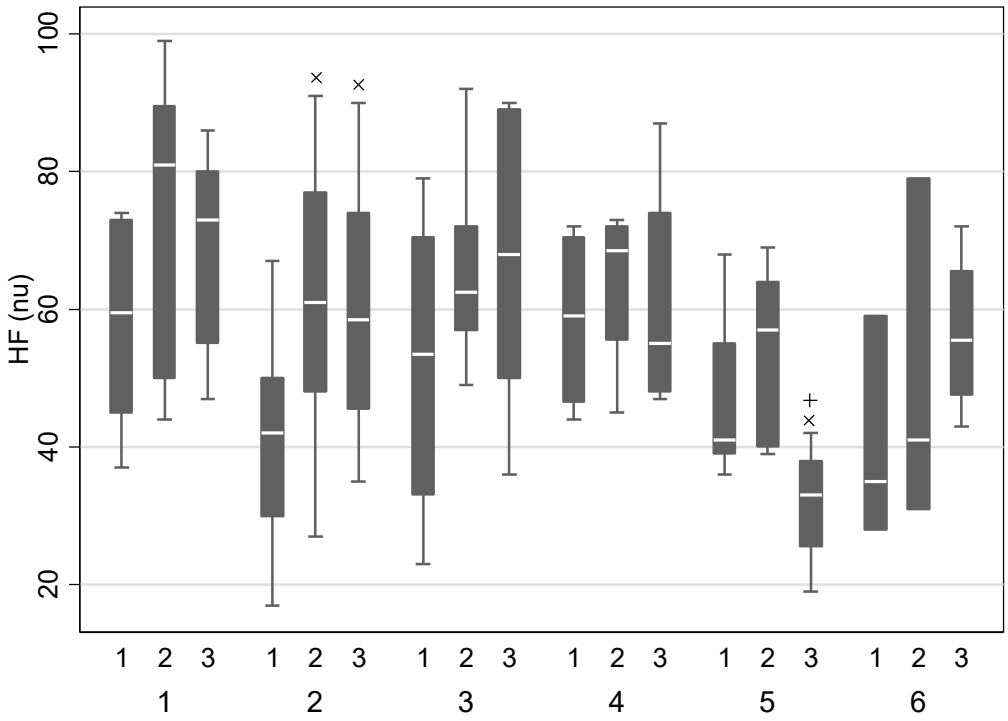
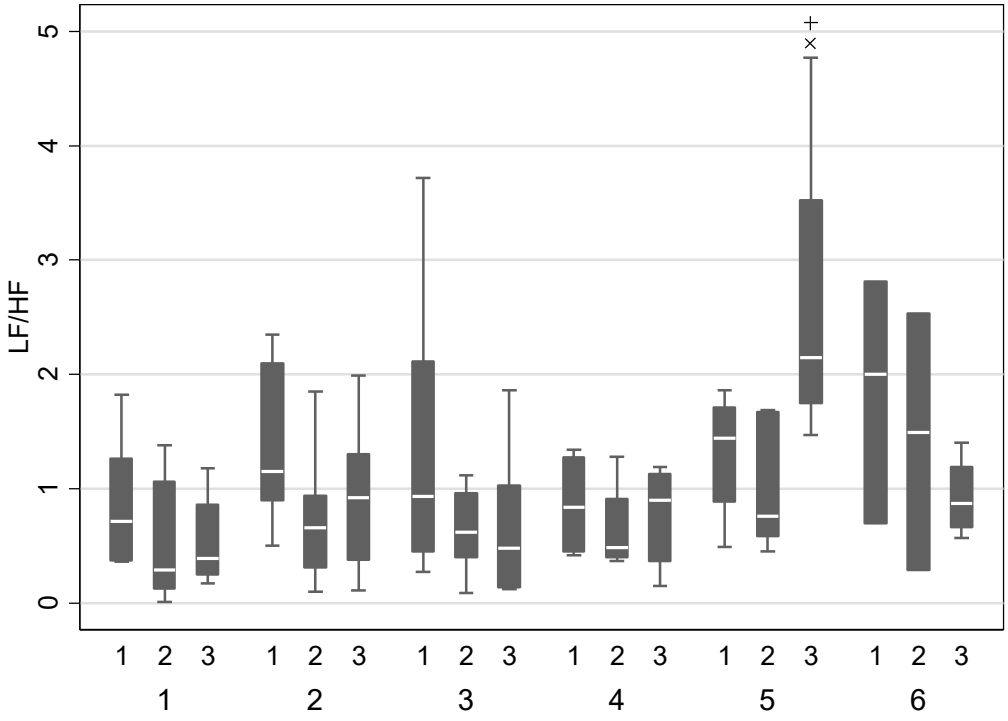


Figure N – Autonomic Balance – LF/HF



Figures L, M and N - Groups: 1=Control; 2=GWI+OVX; 3=OVX Control; 4=Enbrel; 5=COMB; 6=MIF; Time: 1=Pre DFP; 2=After DFP exposure; 3=After drug treatment. **x**= Different from time 1 in the same group; += Different from control group in the same time.