UNIVERSITY OF MANITOBA
Rady Faculty of Health Sciences
Rady College of Medicine

Office of Graduate and
Advanced Degree
Education in Medicine

Student Name: Pegah Afsharinezhad **Date:** 08/04/16

Project Title: Suppression of Doxorubicin and Hypoxia Induced Mitochondrial Perturbations and Necrotic Cell Death of Ventricular Myocytes by the Polyphenolic Compound Ellagic Acid

Primary Supervisor Name: Dr. Lorrie Kirshenbaum

Department: Cardiac Gene Biology

Co-Supervisor Name:

Department:

Summary (250 words max single spaced):

Ellagic acid (EA), is a polyphenolic compound with strong antioxidant properties, however, the effects of EA on cardiac cell death have not been formally investigated. In this report, we provide new exciting evidence that EA suppressed mitochondrial perturbations and cell death of cardiac myocytes mediated by the chemotherapy drug doxorubicin (Dox) or hypoxia (HPX). Cells treated with Dox or subjected to HPX exhibited a marked increase in the inducible death protein Bcl-2 Nineteen Kilodalton Interacting protein 3 (Bnip3). This coincided with mitochondrial perturbations including increased reactive oxygen species (ROS), loss of mitochondrial membrane potential, increased mitochondrial fission and reduced cell viability compared to vehicle treated normoxic control cells. Importantly cardiac myocytes treated with Dox in the presence of EA were indistinguishable from vehicle treated control cells displaying normal mitochondrial morphology, reduced ROS, and preserved mitochondrial membrane potential. Interestingly, mitochondrial targeting of Bnip3 in cells treated with Dox was markedly decreased by EA. This was accompanied by a concomitant reduction in cardiac cell death and increased cell viability. Hence, the present findings provide the first direct evidence that EA is sufficient to suppress mitochondrial injury and cell death of cardiac myocytes induced by Dox and hypoxia by a mechanism that impinges upon the death protein Bnip3. Our exciting data further suggest that by mitigating the cardiotoxic effects of Dox, adjunctive therapy with EA may provide a therapeutic advantage to cancer patients undergoing anthracycline treatment.

Acknowledgments (choose a or b):
a) I gratefully acknowledge the support of the following sponsor: _____

b) I gratefully acknowledge the funding support from one or more of the following sponsors;

H.T. Thorlakson Foundation
Dean, College of Medicine
Research Manitoba
Vice-Dean, Research Rady FHS
Health Sciences Centre Research Foundation
Heart and Stroke Foundation

Student Signature Primary Supervisor Signature

INTRODUCTION:

Malignant neoplasms and cardiovascular diseases have been the two leading causes of all-cause mortality (1). Chemotherapeutic agents such as doxorubicin (Dox) from the anthracycline family, has been the mainstay of treatment for many cancer types including breast carcinoma, gynecologic carcinoma, Hodgkin's disease, non-Hodgkin's lymphoma, thyroid carcinoma, squamous cell carcinoma and gastric carcinoma. However use of this chemotherapeutic treatment comes at a high cost - a patient undergoing Dox chemotherapy is at an extensive risk for deterioration of his or her cardiovascular health in both acute and chronic ways. Acute cardiac events include ECG abnormalities such as non-specific ST-T wave changes; tachyarrhythmia, bradycardia and bundle-branch block which do not necessitate discontinuation of the therapy. However, delayed cardiotoxicities such as reduced left ventricular ejection fraction and/or signs and symptoms of congestive cardiac failure can develop within 2 to 3 months or even years after the treatment (2).

In some cases an available treatment for Dox cardiotoxicity, is dexrazoxane - an iron-chelator that is given to patients receiving a cumulative dose of 300 mg/m2 of Dox. While this drug may suppress some of adverse effects of Dox, it comes with its own detriments. Most importantly, dexrazoxane interferes with the antitumor activity of Dox. It can cause secondary malignancies such as acute myeloid leukemia (AML). It may add to the myelosuppression caused by the chemotherapeutic agent. Moreover, it can cause fetal harm in pregnant women. In terms of safety, this drug has not been studied in geriatric population (>65 years of age), and is not recommended for pediatric population (<18 years of age). Due to its side effects on the body, hematologic, cardiac, hepatic, and renal functions should be monitored during the treatment (3). Thus, while dexrazoxane reduces cardiotoxic effects of Dox, it is not an ideal therapy due to its side effects and that it compromises the efficacy of the antitumor medication.

Cardiovascular diseases including myocardial infarction have a great effect on the human population. Its adverse consequences stem mainly from the inability of cardiac cells to actively regenerate when damaged in sufficient numbers to preserve cardiac function (4). While medications such ACE inhibitors, ARBs, and beta-blockers are used as secondary prevention against ischemic heart disease and treatment of myocardial infarction, consider how game-changing it would be if improved primary prevention mechanisms were available to mitigate the cardiotoxic effects of hypoxia?

Hence, as a first step towards developing new therapies and treatments that protects the heart against cellular injury, is to understand the pathogenesis of the injury at the molecular level. In this regard, the mitochondrion has been identified as a central regulator of cell death through apoptosis and necrosis (4). Mitochondrial disruption specifically from increased levels of ROS has been reported to be the primary cause of cardiac malfunction and subsequent cell death (5). Mitochondria are involved with the function of aerobic respiration where the electron transport chain on the inner mitochondrial membrane reduces oxygen to create ATP and water. During normal conditions, this process is tightly regulated to minimize electron leakage and ROS production (6, 7). In contrast, this process is disrupted during ischemia or hypoxia, where superoxide anions, H2O2 and other ROS result in oxidative stress injury. This is especially important in the context of adult myocardium which exhibits a meager and limited capacity for self-renewal after injury. Hence the inability of cardiac myocytes to actively regenerate coupled with inappropriate cardiac cell death is postulated as a

primary underlying cause of pathologic ventricular remodeling and cardiac failure following Dox treatment (8). Many theories, such as increased ROS production, mitochondrial dysfunction, abnormal iron-handling and contractile failure have been suggested to contribute to Dox cardiotoxicity (9,10,11). Moreover, certain transcription factors involved in the regulation of cell death are reportedly altered in cells treated with Dox (12-15).
Notably, previous work by our lab has shown that Bcl-2-like 19kDa-interacting protein 3 (Bnip3) plays a crucial role in disrupting mitochondrial function and promoting cell death of cardiac myocytes during Dox treatment and hypoxic injury (16,17). Additionally, *in vitro* and vivo, studies have revealed that Bnip3 is a molecular effector of Dox-induced cardiotoxicity. Indeed, previous work by our laboratory demonstrated that Dox elicits mitochondrial injury by interfering with mitochondrial uncoupling protein 3 (UCP3) and cytochrome c oxidase subunit 1(COX1) interactions on mitochondrial inner membrane which was attributed to mitochondrial targeting of Bnip3 (16).

Bnip3 plays a critical role in provoking mitochondrial perturbations on the inner mitochondrial membrane leading to reactive oxygen species (ROS), mitochondrial permeability transition pore opening and loss of $\Delta\Psi m$ of cardiomyocytes (16,17). Genetic ablation of Bnip3 or mutations of Bnip3 defective for mitochondrial targeting were each sufficient to abrogate mitochondrial injury and cell death induced by Dox (16). These studies established Bnip3 as critical mediator of Dox induced mitochondrial injury and cell death of cardiac myocytes.

Ellagic acid (EA), is a naturally occurring polyphenolic compound, which is found in vegetables, nuts, and fruits such as berries and pomegranates (18,19). In a number of pathologies, ellagitinins, which are metabolized to EA have been shown to suppress oxidative stress injury (20,21,22), as well as Bnip3-induced cell death. However the underlying mechanism is not known. Since inner mitochondrial membrane defects induced by Dox lead to increased ROS, expression of Bnip3 and cell death, we reasoned that EA as an adjunctive therapy may provide therapeutic benefit by mitigating the mitochondrial injury and cell death induced by Dox. In this report, we provide compelling new evidence that EA suppresses mitochondrial injury and necrotic cell death of cardiac myocytes induced by Dox by a mechanism that impinges upon Bnip3.

MATERIALS AND METHODS

Cell Culture

Postnatal rat cardiac myocytes were isolated from 1-2 day old Sprague-Dawely rats and subjected to primary culture as previously described. Cells were treated with EA (10μM Sigma), Dox (10 μM Pfizer, Canada) or subjected to HPX for 18 hours (16). EA was dissolved in DMSO in a warm bath. HPX, cells were kept in HPX chamber for up to 18 hours as we previously described (17, 23).

Cell Viability and Immunostaining

Postnatal ventricular cardiomyocytes were stained with the vital dyes calcein acetoxymethylester (Calcein-AM) and ethidium homodimer-1 (each 2 μM) to visualize live (green) and dead (red) cells, respectively by epifluorescence microscopy using AX-70, Olympus fluorescence microscope under the magnification of 40x at room temperature in DMEM/F12 serum-free medium and images were taken by Image-Pro plus 5.0 software. At least >200 cells were counted using replicates of n=2-3 for each condition tested. Data are expressed as mean ±SEM percent dead cells from control.

Mitochondrial Injury and Mitochondrial Fission Analysis
Mitochondrial membrane potential was assessed using 50nM of the fluorescent dye, tetra-methylrhodamine methyl ester perchlorate (TMRM) (Molecular Probes, Eugene Oregon). ROS were monitored using 2.5μM Dihydroethidium (Molecular Probes, Eugene Oregon). Red fluorescence demarks ROS. Cells were visualized by epifluorescence microscopy as described (16). For mitochondrial morphology assessment, cardiac myocytes were stained with Mitotracker Red CMXRos dye. Images were acquired using research fluorescence microscope (Carl Ziess AX10 observer SD) under the magnification of 630x at room temperature using ZEN software (Carl Zeiss).

Using Image J software, raw images were binarized and mitochondria were subjected to particle analyses to obtain values for form factor (FF: perimeter2/4π·Area), which is a measure for both length and branching. Minimal value of 1 indicates a small perfect circle, an increase in the values indicate elongation and branching (23).

Western Blot analysis
Western Blot analysis was performed for protein expression on cell lysate extracted from cardiac myocytes. Protein extracts were resolved on denaturing SDS PAGE gels transferred to nitrocellulose membranes. The Filters were probed with primary Bnip3 antibody generated in house as reported (25). DRP1, P-DRP antibodies were purchased from Cell Signaling Technology and α-actin antibody was from Sigma Aldrich. All antibodies were used at 1:1000 dilutions in 2% non-fat milk containing 0.1% TBS-T overnight at 4°C. Bound proteins were detected using secondary anti-mouse or rabbit antibodies conjugated to horse radish peroxidase by enhanced ECL (Pharmacia).

Statistical Analysis
Multiple comparisons between groups were tested by one-way ANOVA. Bonferroni post hoc tests were used to determine difference among groups. Unpaired two tailed Students-t test was used to compare mean difference from control. Differences were considered to be statistically significant to a level of p value <0.05. In all cases data was obtained from at least n=3-4 independent myocyte isolations using n=2 replicates for each condition tested.

RESULTS

Ellagic Acid Suppresses Doxorubicin- and HPX-Induced Reactive Oxygen Species
Previous work by our laboratory has established that Dox and HPX result in lethal changes to the cell at the organelle and molecular level. As shown in figure 1, ultrastructural analysis of hearts of mice treated with either saline (Panels A and C) or Dox (B and D) are depicted. As shown in the figure, in contrast to vehicle treated controls which displayed normal cardiac architecture, disrupted sarcomeres, mitochondrial defects such as swelling, loss of cristae, and vacuolization was readily observed in hearts of mice treated Dox. To understand the mechanisms underlying mitochondrial defects, we conducted *in vitro* cell culture studies. Consistent with mitochondrial defects *in vivo*, we observed mitochondrial defects such as increased ROS, loss of membrane potential in Dox or HPX treated cardiomyocytes *in vitro*. We next explored whether EA would suppress the cardiac toxic effects of Dox. As shown by epifluorescence microscopy in figure 2 panels A and B, in contrast to vehicle treated control cells, cells treated with Dox displayed a marked increase in ROS production as indicted by increased dihydroethidium fluorescence (red) cells. Importantly, dox- induced

ROS production was suppressed in cardiac myocytes treated with EA. In addition, EA also suppressed ROS production in cells subjected to HPX, verifying the cardio protective effects of EA on mitochondrial ROS production were not restricted to Dox induced injury.

Ellagic Acid Suppresses Doxorubicin- and Hypoxia-Induced Loss of Mitochondrial Membrane Potential

Based on the above findings, we next tested whether EA would preserve the membrane potential in cells treated with Dox or subjected to HPX. For these studies we monitored mitochondrial membrane potential ($\Delta\Psi m$) using tetra methyl rhodamine (TMRM) red fluorescence. As shown in Figure 3 panels A and B, a marked reduction in $\Delta\Psi m$ was observed in cells treated with Dox or cells subjected to HPX. Importantly, however, loss of $\Delta\Psi m$ in cells treated with Dox or subjected to HPX was normalized to control levels by EA.

Ellagic Acid Suppresses Doxorubicin-Induced Mitochondrial Fission

Since loss of mitochondrial $\Delta\Psi m$ can promote mitochondrial fission resulting in cell death, we explored whether mitochondrial fission was induced in cells treated with Dox. As shown in figure 4 panels A and B, mitochondrial fission was markedly increased in cells treated with Dox. This is shown graphically by the histogram which represents the aspect ratio of mitochondrial length and width as an index of mitochondrial fission. Additionally, dynamin-related protein 1 (DRP1), a mediator of mitochondrial fission (32), and its activated phosphorylated form, P-DRP1 was increased in Dox-treated cells as shown by Western blot analysis (Figure 5, A). Importantly, EA suppressed Dox induced DRP1 activation and mitochondrial fission (4A-B, 5A).

Ellagic Acid Supresses Doxorubicin and Hypoxia Mediated Cell Death

It has been established that Dox and HPX cause cardiac cell death – which eventually leads to heart failure (8). It was our main objective to test whether EA is sufficient to protect cardiac myocytes against the cytotoxic effects of Dox or HPX. As shown in figure 6, panels A and B, a dramatic reduction in cell death induced by Dox or HPX was observed in cells treated with EA. Furthermore, we also observed that EA suppressed cell death of mouse embryonic fibroblasts (MEF) induced by either Dox or HPX indicating that cytoprotective effects of EA are not restricted to cardiac myocyets (Figure 7 A-B).

Ellagic Acid Suppresses Expression of Bnip3 in Cardiac Myocytes Treated with Doxorubicin or Subjected to Hypoxia

Earlier work established that Bnip3 was essential for triggering mitochondrial injury in cardiomyocytes resulting in necrosis (16,17). Under normal conditions, Bnip3 is predominantly localized to cytoplasm, but targets the mitochondrion during cell stress where it disrupts mitochondrial function and provokes cell death. To test the underlying mechanism by which EA suppresses mitochondrial perturbations and cell death, we assessed whether EA influences Bnip3 activity. As shown by Western blot analysis in Figure 5 A-B, Bnip3 expression was markedly increased in cells treated with Dox- or subjected to HPX. Interestingly, Dox or HPX induced Bnip3 expression was suppressed by EA treatment. These findings, strongly suggest that EA suppresses cell death induced by Dox by a mechanism that impinges on the expression of Bnip3.

DISCUSSION

In this report, we provide evidence that EA can be a profound cardioprotective compound that preserves the health of neonatal ventricular cardiomyocytes when exposed to the chemotherapeutic drug – Dox, or HPX – a simulation of ischemia. EA is sufficient to suppress mitochondrial distress and cell death during an oxidative stress injury. It is even of more significance that such cardioprotective effects are related to the Bnip3 protein, which has not been previously reported. Although oxidative stress is an established feature of Dox and hypoxic injury, the underlying mechanism by which EA is able to save the cells remains to be discovered. We have previously established in our lab that Bnip3 is essential for mitochondrial perturbations as it binds to the mitochondrial membrane and disrupts the electron transport chain in Dox or HPX treated cardiomyocytes. This results in a loss of transmembrane potential, release of oxygen species and cell death in ventricular myocytes in HPX and Dox toxicity (16).

During normal basal conditions, the Bnip3 promoter is strongly suppressed by inhibitory histone deacetylase complexes, which represses Bnip3 gene transcription and cell death (33). This is in contrast to oxidative stress during Dox and HPX conditions as Bnip3 promoter becomes transcriptionally activated (16).Since Bnip3 activation leads to mitochondrial damage and cell death, disruption in such an account would be of significant therapeutic value. This is especially important when EA is able to significantly decrease mitochondrial damage induced by Dox or HPX, both of which are known to involve Bnip3 (18). This strongly suggests that EA antagonizes the effects of Bnip3-mediated mitochondrial injury rather than it being just a simple ROS scavenger as previously reported (14, 15, 18).

Currently, the underlying mechanism by which EA blocks mitochondrial targeting of Bnip3 is not known, but we speculate that the two are related directly or indirectly through one or more factors. It is suggested that EA can affect and disrupt the mitochondrial targets of Bnip3 post-translationally. This area remains for future investigations.

The possibility that the EA has cytoprotective effects besides suppressing mitochondrial injury induced by Bnip3 cannot be excluded, but may also prompt secondary properties related to production of ROS. However, it is hard to deny the fact that mitochondrial injury and ROS production induced by Bnip3 were substantially suppressed by EA arguing against such possibility and shining a light on a unique signaling pathway that mechanically relates EA and Bnip3 to cell survival. Such property of EA can be of significant benefit to suppressing mitochondrial injury and cell death of cardiac myocytes in patients undergoing Dox treatment or during myocardial infarction.

The present study demonstrates that EA has a cardioprotective effect against Dox and HPX. Notably, our preliminary research data suggests that it does not interfere with the anti-tumor properties of Dox. Hence, this unique feature of EA highlights its use as a promising adjunctive therapy for mitigating the cardiomyopathy in cancer patients undergoing Dox treatment.

ABBREVIATIONS

EA, Ellagic acid; DOX, Doxorubicin; ROS, Reactive Oxygen Species; $\Delta\Psi m$, Membrane potential; H_2O_2, Hydrogen peroxide; Calcein-AM, Calcein acetoxymethylester; TMRM,

Tetra-methylrhodamine methyl ester perchlorate; FF, Form factor; SDS PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS-T, Tris Buffered Saline with Tween 20; ECL, Enhanced chemiluminescence.

ACKNOWLEDGEMENT

I wish to express my gratitude to my supervisor Lorrie Kirshenbaum for his guidance, encouragement, support, and energy throughout my project as well as our lab members: Rimpy Dhingra, Vicky Margulets, Floribeth Aguilera, Matthew Guberman, Leah Bernhardt, Abhinav Dhingra, Alexis Nemeth, Sara London, Matthew Love, and Kairee Ryplanski. I'd also like to thank my parents for their unconditional support.

CONFLICT OF INTEREST

No conflict of interest.

REFERENCES

1. The 10 leading causes of death, 2011 [Internet]. Statcan.gc.ca. 2015 [cited 5 July 2016]. Available from: http://www.statcan.gc.ca/pub/82-625-x/2014001/article/11896-eng.htm

2. Doxorubicin [Internet]. E-therapeutics.ca.uml.idm.oclc.org. 2016 [cited 6 July 2016]. Available from: http://www.etherapeutics.ca.uml.idm.oclc.org/search

3. Dexrazoxane [Internet]. E-therapeutics.ca.uml.idm.oclc.org. 2016 [cited 6 July 2016]. Available from: http://www.etherapeutics.ca.uml.idm.oclc.org/searc

4. 2. Mughal W, Dhingra R, Kirshenbaum L. Striking a Balance: Autophagy, Apoptosis, and Necrosis in a Normal and Failing Heart. Curr Hypertens Rep. 2012;14(6):540-547.

5. Singal PK, Kirshenbaum LA. A relative deficit in antioxidant reserve may contribute in cardiac failure. Can J Cardiol. 1990 Mar;6(2):47-9.

6. Kirkinezos IG, Moraes CT. Reactive oxygen species and mitochondrial diseases. Semin Cell Dev Biol. 2001 Dec;12(6):449-57.

7. Nicholls DG. Spare respiratory capacity, oxidative stress and excitotoxicity. Biochem Soc Trans. 2009 Dec;37(Pt 6):1385-8.

8. Singal PK, Iliskovic N. Doxorubicin-induced cardiomyopathy. N Engl J Med. 1998 Sep 24;339(13):900-5.

9. Ichikawa Y, et al. Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. J Clin Invest. 2014;124(2):617–630.

10. Simůnek T, et al. Anthracycline-induced cardiotoxicity: Overview of studies examining the roles of oxidative stress and free cellular iron. Pharmacol Rep. 2009;61(1):154–171.

11. Berthiaume JM, Wallace KB. Adriamycin-induced oxidative mitochondrial cardiotoxicity. Cell Biol Toxicol. 2007;23(1):15–25.

12. Abello PA, Fidler SA, Buchman TG. Thiol reducing agents modulate induced apoptosis in porcine endothelial cells. Shock. 1994 Aug;2(2):79-83.

13. Assaly R, de Tassigny Ad, Paradis S, Jacquin S, Berdeaux A, Morin D. Oxidativestress, mitochondrial permeability transition pore opening and cell death during hypoxia-reoxygenation in adult cardiomyocytes. Eur J Pharmacol. 2012 Jan30;675(1-3):6-14.

14. Kannan K, Jain SK. Oxidative stress and apoptosis. Pathophysiology. 2000 Sep;7(3):153-163.

15. McCord JM. Superoxide radical: controversies, contradictions, and paradoxes. Proc Soc Exp Biol Med. 1995 Jun;209(2):112-7.

16. Dhingra R, Margulets V, Chowdhury SR, Thliveris J, Jassal D, Fernyhough P, Dorn GW 2nd, Kirshenbaum LA. Bnip3 mediates doxorubicin-induced cardiac myocyte necrosis and mortality through changes in mitochondrial signaling. Proc Natl Acad Sci U S A. 2014 Dec 23;111(51):E5537-44.

17. Regula KM, Ens K, Kirshenbaum LA. Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. Circ Res. 2002 Aug 9;91(3):226-31.

18. Almeida IF, Fernandes E, Lima JL, Valentão P, Andrade PB, Seabra RM, Costa PC, Bahia MF. Oxygen and nitrogen reactive species are effectively scavenged by Eucalyptus globulus leaf water extract. J Med Food. 2009 Feb;12(1):175-83.

19. Hwang JM, Cho JS, Kim TH, Lee YI. Ellagic acid protects hepatocytes from damage by inhibiting mitochondrial production of reactive oxygen species. Biomed Pharmacother. 2010 Apr;64(4):264-70.

20. Mishra K, Girdhani S, Bhosle S, Thulsidas S, Kumar A. Potential of radiosensitizing agents in cancer chemo-radiotherapy. J Can Res Ther. 2005;1(3):129.

21. Kabiraj P, Marin J, Varela-Ramirez A, Zubia E, Narayan M. Ellagic Acid Mitigates SNO-PDI Induced Aggregation of Parkinsonian Biomarkers. ACS Chem Neurosci. 2014;5(12):1209-1220.

22. Salimi A, Roudkenar M, Sadeghi L, Mohseni A, Seydi E, Pirahmadi N et al. Ellagic acid, a polyphenolic compound, selectively induces ROS-mediated apoptosis in cancerous B-lymphocytes of CLL patients by directly targeting mitochondria. Redox Biology. 2015;6:461-471.

23. Gurevich R, Regula K, Kirshenbaum L. Serpin Protein CrmA Suppresses Hypoxia-Mediated Apoptosis of Ventricular Myocytes. Circulation. 2001;103(15):1984-1991.

24. Yousef AI, El-Masry OS, Yassin EH. The anti-oncogenic influence of ellagic acid on colon cancer cells in leptin-enriched microenvironment. Tumour Biol. 2016 Jul 26.

25. Núñez-Sánchez MÁ, Karmokar A, González-Sarrías A, García-Villalba R,Tomás-Barberán FA, García-Conesa MT, Brown K, Espín JC. In vivo relevant mixed urolithins and ellagic acid inhibit phenotypic and molecular colon cancer stem cell features: A new potentiality for ellagitannin metabolites against cancer.Food Chem Toxicol. 2016 Jun;92:8-16

26. Shaw J, Yurkova N, Zhang T, Gang H, Aguilar F, Weidman D et al. Antagonism of E2F-1 regulated Bnip3 transcription by NF- B is essential for basal cell survival. Proceedings of the National Academy of Sciences. 2008;105(52):20734-20739.

27. Naiki-Ito A, Chewonarin T, Tang M, Pitchakarn P, Kuno T, Ogawa K, Asamoto M, Shirai T, Takahashi S. Ellagic acid, a component of pomegranate fruit juice, suppresses androgen-dependent prostate carcinogenesis via induction of apoptosis. Prostate. 2015 Feb;75(2):151-60.

28. Rocha A, Wang L, Penichet M, Martins-Green M. Pomegranate juice and specific components inhibit cell and molecular processes critical for metastasis of breast cancer. Breast Cancer Res Treat. 2012 Dec;136(3):647-58

29. Cerdá B, Espín JC, Parra S, Martínez P, Tomás-Barberán FA. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. Eur J Nutr. 2004 Aug;43(4):205-20.

30. Cerdá B, Llorach R, Cerón JJ, Espín JC, Tomás-Barberán FA. Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. Eur J Nutr. 2003 Jan;42(1):18-28.

31. Espín JC, González-Barrio R, Cerdá B, López-Bote C, Rey AI, Tomás-Barberán FA. Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. J Agric Food Chem. 2007 Dec 12;55(25):10476-85..

32. Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, Youle RJ. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell. 2001 Oct;1(4):515-25.

33. Shaw, J., Zhang, T., Rzeszutek, M., Yurkova, N., Baetz, D., Davie, J., & Kirshenbaum, L. (2006). Transcriptional Silencing of the Death Gene BNIP3 by Cooperative Action of NF- B and Histone Deacetylase 1 in Ventricular Myocytes. *Circulation Research*, *99* (12), 1347-1354

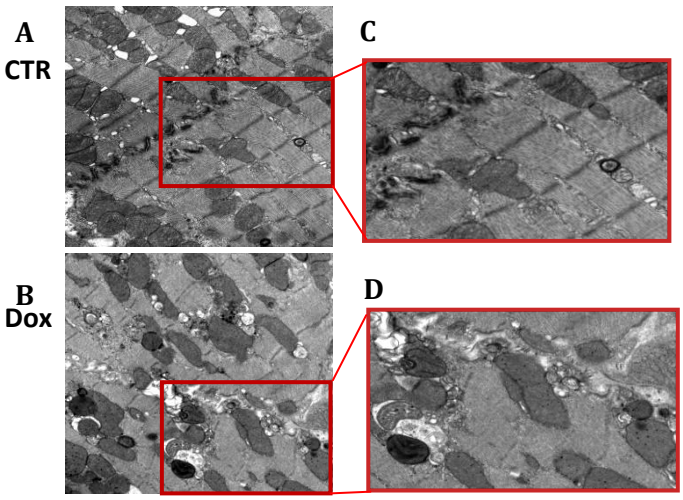

Figure 1. Electron microscopy of murine cardiac muscle. Saline-treated cardiomyocytes showing normal ultrastructures **(A&C)**. Dox-treated cardiomyocytes (single i.p injection, 20 mg/kg) at 10 day post-injection **(B)**. Magnified section **(D)** shows disrupted sarcomeres, loss of cristae, mitochondrial swelling, and vacuolization. Red arrows show membrane structures suggestive of autophagosomes. (Magnification 5800x)

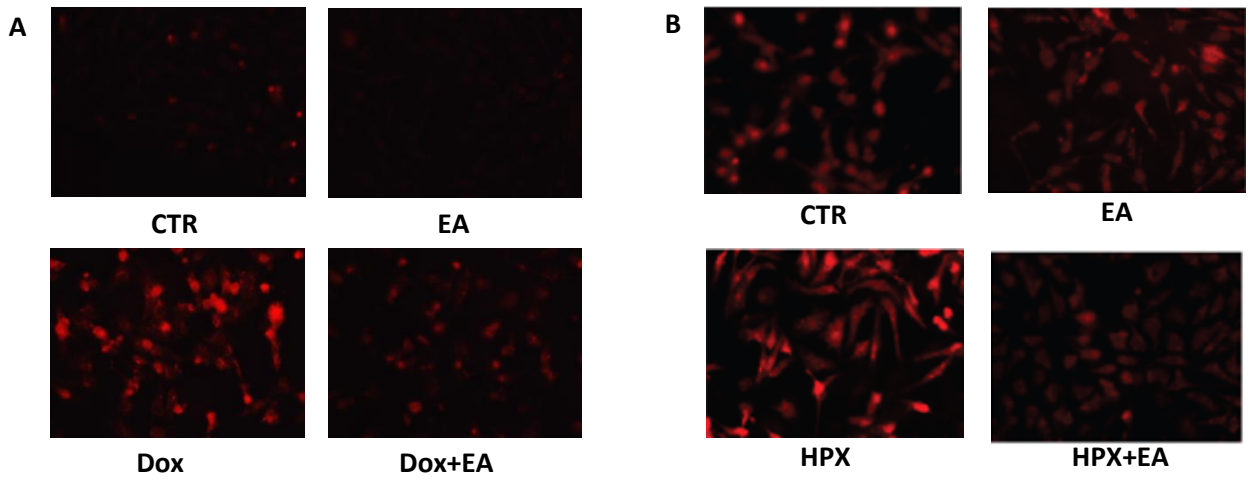

Figure 2. Epifluorescence microscopy detecting ROS in cardiomyocytes as assessed by dihydroethidium (red). In **(A)** Dox (10μM)-treated cells have more ROS compared to control. Dox and Ellagic acid (10μM) treatment shows substantial reduction in ROS compared to the Dox treatment. In **(B)** ROS is measured in hypoxia-treated (18hrs.) myocytes ROS is increased in hypoxia condition and reduced when ellagic acid was added.

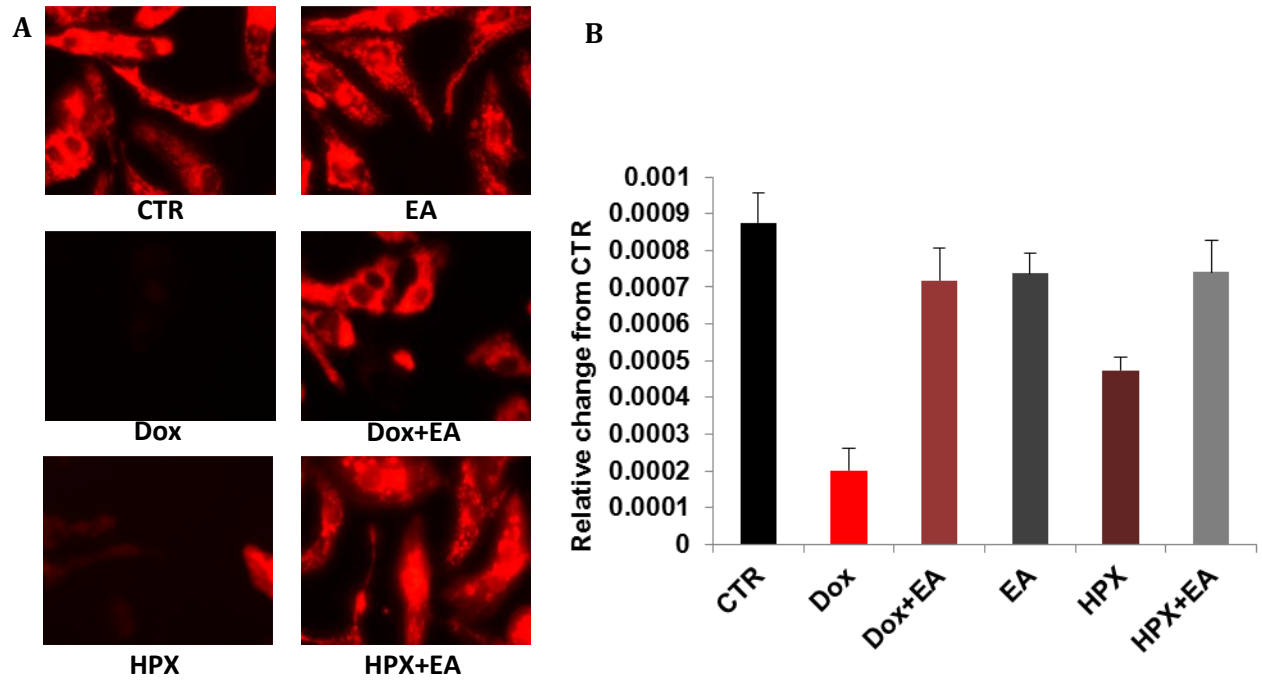

Figure 3. Mitochondrial membrane potential (ΔΨm) assessed by TMRM (red) in Dox and HPX-treated myocytes. **(A)** ΔΨm is remarkably reduced in Dox and HPX-treated cells and greatly restored by ellagic acid. **(B)** Bar chart for quantitative data for conditions shown in panel **A** - relative change of ΔΨm from control in Dox, hypoxia, with addition of ellagic acid is measured. Data was obtained from n=3-4 independent myocyte isolations.

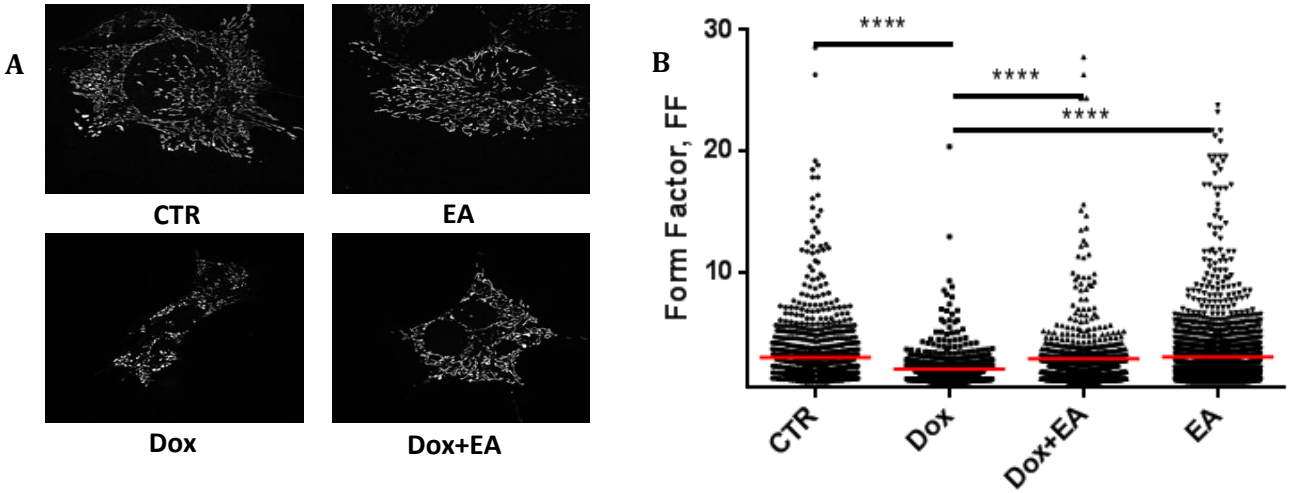

Figure 4. Epifluorescence microscopy of mitochondrial morphology in Dox-treated cardiomyocytes with or without ellagic acid stained with mitotracker (shown as white) **(A)**. The dot plots **(B)** show the median values (line) of FF (form factor) for all mitochondria of the cells analyzed per condition. Star shows significant values with p<.05.

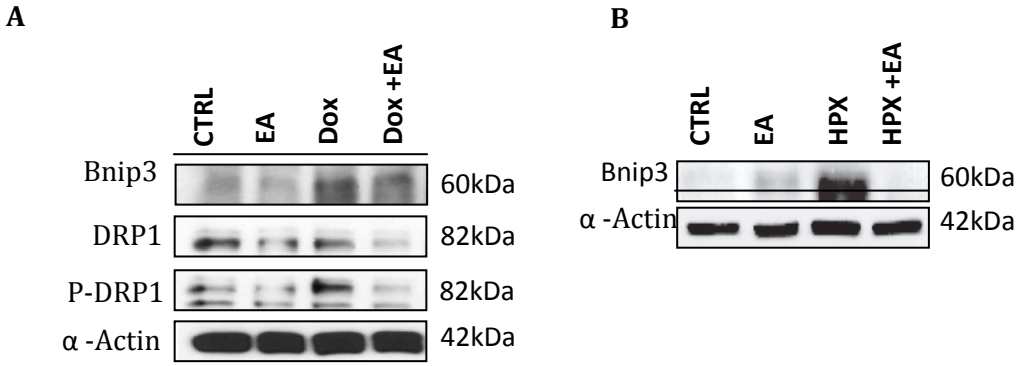

Figure 5. **Panel A** shows western blots for cardiomyocytes treated with Dox (10μM) in presence or absence of ellagic acid probed for antibodies against Bnip3, DRP1, P-DRP1 and α –Actin. **Panel B** shows anti-Bnip3 and anti- α –Actin probing in cardiomyocytes treated with hypoxia with or without ellagic acid.

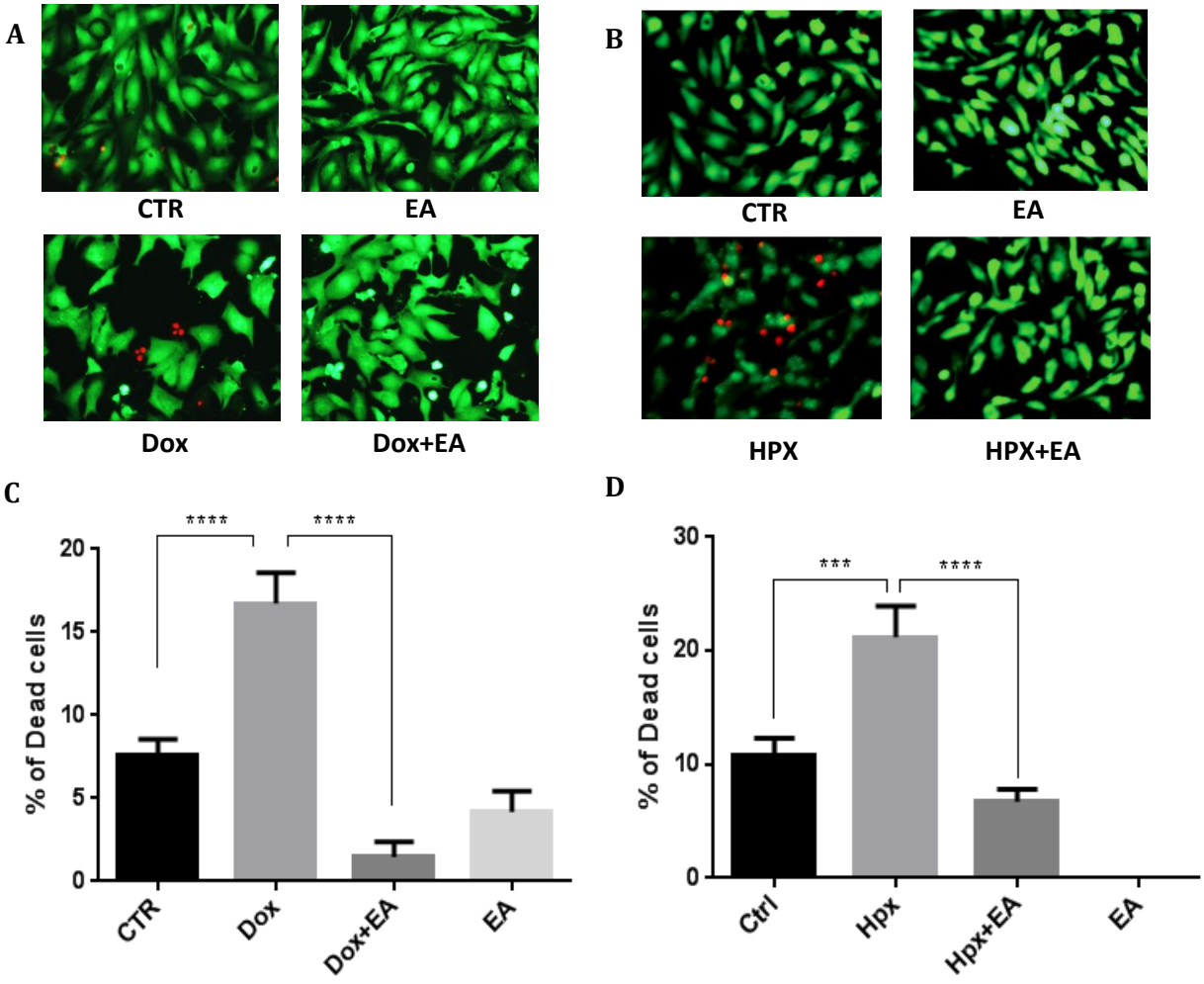

Figure 6. Panel A and B epifluorescence microscopy of ventricular cardiomyocytes stained for vital dyes calcein-AM and ethidium-homodimer for detection of live (green) and dead cells (red). **Panel A** cells are treated with Dox (10μM) in presence or absence of ellagic acid. **Panel B** shows cardiomyocytes treated with hypoxia (18 hrs.) in the presence or absence of ellagic acid. **Panel C**, and **D** are bar charts representing quantitative data for A and B respectively. The data is expressed as mean +/- SEM.

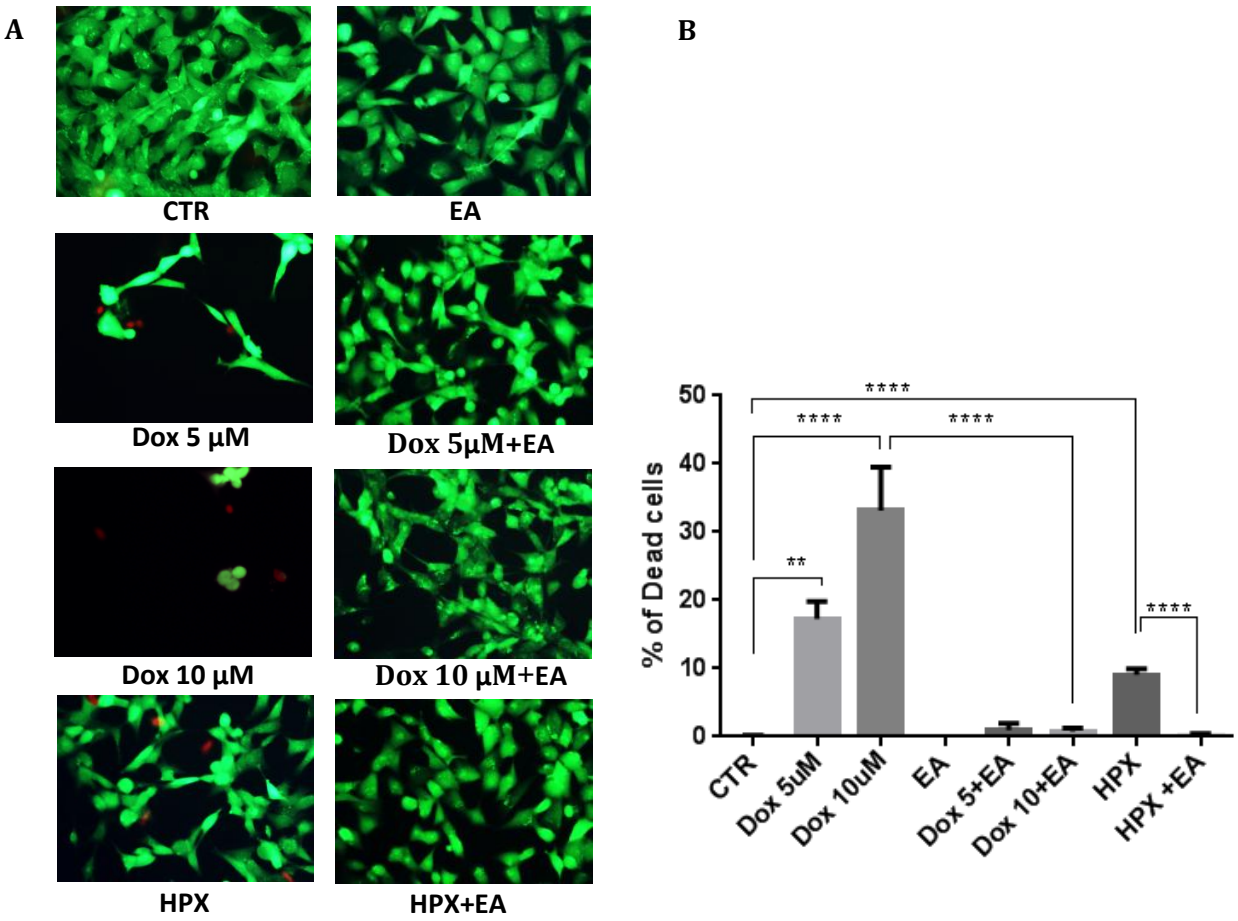

Figure 7. **Panel A** shows epifluorescence microscopy of wildtype MEF cells stained for vital dyes calcein-AM and ethidium-homodimer for detection of live (green) and dead cells (red). Cells are treated with Dox (5µM), Dox (10µM) or hypoxia (18 hrs.) in presence or absence of ellagic acid. **Panel B** is bar chart representing quantitative data for A. The data is expressed as mean +/- SEM.