Sexual Dimorphism of Doxorubicin-Mediated Cardiotoxicity: Potential Role of Energy Metabolism Remodeling

Moulin et al: Doxorubicin Cardiotoxicity Is More Severe in Males

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DOI: 10.1161/CIRCHEARTFAILURE.114.001180

Abstract

Background—Cardiovascular diseases (CVD) are the major cause of mortality among both men and women with a lower incidence in women before menopause. The clinical use of doxorubicin, widely used as an antineoplastic agent, is markedly hampered by severe cardiotoxicity. Even if there is a significant gender difference in incidence of CVD at the adult stage, it is not known whether a difference in doxorubicin-related cardiotoxicity between men and women also exists. The objective of this work was to explore the cardiac side effects of doxorubicin in adult rats and decipher if signaling pathways involved in cardiac toxicity differ between sexes.

Methods and Results—After 7 weeks of doxorubicin (2mg/kg/week), males developed major signs of cardiomyopathy with cardiac atrophy, reduced left ventricular ejection fraction (LVEF) and 50% mortality. In contrast, no female died and their LVEF was only moderately affected. Surprisingly, neither global oxidation levels nor the anti-oxidant response nor the apoptosis signaling pathways, were altered by doxorubicin. However, level of total adenosine-mono-phosphate-activated protein kinase (AMPK) was severely decreased only in males. Moreover, markers of mitochondrial biogenesis and cardiolipin content were strongly reduced only in males. To analyze the onset of the pathology, maximal oxygen consumption rate of left ventricular permeabilized fibers after 4 weeks of treatment, was reduced only in doxorubicin-treated males.

Conclusions—Altogether these results clearly evidence sex differences in doxorubicin toxicity. Cardiac mitochondrial dysfunction and AMPK appear as critical sites of sex differences in cardiotoxicity as evidenced by significant statistical interactions between sex and treatment effects.

Key Words: sex-specific, heart failure, mitochondria, AMPK, PGC-1alpha, anthracycline
Cardiovascular diseases (CVDs) are the major cause of death globally. A number of studies show gender differences in morbidity and treatment efficacy in CVD patients; thus highlighting the need for gender specific therapy.\(^1\),\(^2\) It is well accepted that women develop less CVD or maintain better cardiac function than do men at least before menopause, in part because of differences in the hormonal status, but not exclusively.\(^2\),\(^3\) Nevertheless, sex differences in cardiovascular pathophysiology have been poorly studied so far.\(^3\)

Anthracyclines remain among the most potent anticancer drug. They are an important component of numerous chemotherapy protocols of both hematological malignancies and solid tumors. However, their clinical use is hampered by the risk of severe cardiotoxicity which may lead to a progressive cardiomyopathy that may evolve to congestive heart failure (CHF). Cardiotoxicity refers to deleterious and unwanted side effects of therapeutic compounds on heart function. There are several risk factors for cardiotoxicity induced by anthracyclines such as total cumulative dose, additional treatment, existing cardiomyopathy, age and sex.\(^4\) However, doxorubicin-related gender differences in cardiotoxicity have been under-analyzed. One study showed that male lymphoma patients treated with doxorubicin showed a greater sub-clinical late cardiomyopathy compared to female patients.\(^5\)

Experimental studies lack information as most work has been conducted in males,\(^6\) but some studies suggest that females may develop less severe doxorubicin-induced cardiomyopathy and nephropathy than males.\(^7\),\(^9\) Investigation of protective mechanisms operating in female organisms could be a promising approach in searching for new therapeutic tools.

Deficits of mitochondrial function, biogenesis and energy metabolism have recently emerged as key contributors to heart failure.\(^10\),\(^11\) Interestingly, mitochondrial alterations also seem to be involved in doxorubicin-mediated cardiotoxicity.\(^12\),\(^13\) Moreover, alterations in energy signaling pathways like the AMP-activated protein kinase (AMPK) have been recently shown
to be involved in doxorubicin cardiotoxicity\textsuperscript{12} but possible gender-differences have not yet been evaluated.

The aim of this study was therefore 1) to examine gender-specific doxorubicin cardiotoxicity, 2) to investigate energy metabolism and 3) to identify signaling pathways that could be involved in these differences.

**Methods**

An expanded and detailed method section is available in the supplemental section.

**Animal conditioning**

A chronic model of doxorubicin was chosen by weekly intravenous injection of 2 mg/kg doxorubicin or saline solution during 7 weeks in 16 male and 12 female adult Wistar rats (Janvier Labs). Analysis of doxorubicin 2 hours after i.v. injection showed similar level of doxorubicin in hearts from male and female rats (Male=7.9±0.6; Female=8.2±1.6 ng doxorubicin/mg dw). Because of ethical concerns of doxorubicin-treated males at 7 weeks (20% loss of initial weight, prostration and 50% mortality), the number of rats could not be enlarged. For experiments aimed at characterizing early dysfunction, animals were treated for 4 weeks (20 females and 20 males). Animal experimental procedures were approved by the Animal Ethics Committee of Paris-Sud University. Investigations were done in accordance with European Community legislation relating to the care and use of animals (Directive 2010/63/EU), and the corresponding French legislation (French decree 2013-118 du 1er février 2013).
Statistical analysis

Data are expressed as mean±SEM unless specified. The survival of the animals was analyzed by the Kaplan-Meier method. The weight loss of the animals was analyzed by a two way repeated measures ANOVA (normal distribution (Shapiro-Wilk test) and homogenous variance verified). Two-way ANOVA was used to study the global effect of sex and treatment, followed by Student–Newman–Keuls post hoc test to determine individual differences (normal distribution (Shapiro-Wilk test) and homogenous variance verified). Pearson correlation was performed. P-values (2 sided) are presented to 3 decimal places if significant ant 2 if not. Statistical significance between NT and DOXO group was defined as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***)

Results

Females have better survival and minor body mass loss compared to males after 7 weeks of doxorubicin

After seven weeks of doxorubicin treatment, female rats survived and had normal appearance. However, doxorubicin induced a severe toxicity among males with 50% mortality (Figure 1A). These animals were less active with poor appetite, had a significant weight loss over time (Figure 1B) and slowing of growth together with severe cardiac and splenic atrophy (Table). Both sexes had enlarged liver, pale and swollen kidneys and increase in triglycerides and cholesterol serum content. To note, none of the treated rats presented cardiac hypertrophy. Overall, females were much less affected than males as evidenced by the significant interaction between sex and treatment effects on the body, and heart weight, lung, kidney and tibia length (Table).
Only males present important signs of heart failure after 7 weeks of doxorubicin treatment

Gross examination of treated males revealed the presence of ascites, pleural effusion, and increased lung weight indicating heart failure in surviving animals, suggesting that the sickest hearts may have been missed due to lethality. Left ventricular levels of atrial natriuretic factor (ANF), interleukin-6 (IL-6) and myocyte-enriched calcineurin-interacting protein (McIPI) mRNA, markers of myocardial stress, were more up-regulated in treated males than females (Figure 1C). Histological assessment of male and female heart (Figure 1D and 1E) revealed that while perimyocyte fibrosis was similar in both sexes, total level of fibrosis and reactive fibrosis were more important in doxorubicin-treated males (Figure S1A-C). We noticed inflammatory infiltrate in treated animals but accumulation of vacuoles and important myolysis were specific to treated males (Figure 1E). Echocardiography showed a strong reduction of the fractional shortening (FS) and left ventricular ejection fraction (LVEF) for treated males from 87.5±2.0 to 55.9±0.6%, (p<0.0002) while female LVEF was only moderately affected (86.3±2.3 to 79.0±1.4%, p<0.01) (Figure 2A). Similarly, gender difference after doxorubicin treatment was observed for the systolic LV internal diameter (LVIDs) with an increase only for males. Lower diastolic LVID in females compared to males was not changed by doxorubicin. The interventricular septal wall thickness (IVS) in diastole was similar in both sexes and was not modified by doxorubicin treatment while in males the drug reduced IVS in systole. Cardiac output was different between sexes and decreased only in treated males. All cardiac parameters present significant statistical interactions between sex and doxorubicin effects, except for diastolic parameters and cardiac output normalized to body weight (Figure 2B). These results combined with mortality, anatomical and histological data reveal a sex-related cardiomyopathy difference with a worse cardiac function for doxorubicin-treated males.
Death signaling pathways and oxidative stress are similar in doxorubicin-treated males and females

In order to investigate the mechanism of gender-dependent responses to doxorubicin, we first analyzed cell death. We observed a greater increase in global cell death in male than female hearts (Figure 3A and S1E). However, cardiomyocyte TUNEL staining was similar in both sexes (Figure 3A) but inflammatory infiltrate cells were highly TUNEL positive in treated males. The absence of sex difference in doxorubicin-induced cell death was confirmed in adult cardiomyocytes isolated either from male or female rats (Figure 3B and C). The percentage of viable cells was only 32±4% after 24 hours of doxorubicin treatment compared to 72±2% for non-treated cells and no difference was observed between male or female cardiomyocytes. Doxorubicinol, the main metabolite of doxorubicin, was slightly more toxic as viability was only 15%; once again no sex difference was observed (data not shown).

Furthermore, gene expression of cell death, ER stress and autophagy markers was studied. Tumor necrosis factor alpha (Tnfa). Bcl2, Beclin, Chop and Grp78 did not differ between sexes or following doxorubicin-treatment (Figure 3D). Superoxide dismutase 2 (Sod2) and catalase (Cat) but not glutathione peroxidase 1 (Gpx1) gene expression were also altered in both sexes after doxorubicin treatment (Figure 3E), but no sex difference was observed for these ROS detoxification enzymes. Surprisingly, global protein carbonylation was comparable in all rat groups (Figure 3F and S2), with a slight trend for lower level in females, and higher level in doxorubicin treated animals. Overall, these results suggest that death signaling pathway and oxidative stress are not primarily involved in the sexual dimorphism of doxorubicin cardiotoxicity.
Specific down-regulation of carbohydrate utilization pathway in males after 7 weeks of doxorubicin treatment

Previous studies showed the importance of AMP-activated protein kinase (AMPK) in the cardiotoxicity of anthracyclines, but these were conducted only in males.12, 14 In order to elucidate whether the gender-dependent doxorubicin cardiotoxicity difference could be linked to AMPK signaling, we analyzed the level of total and phosphorylated AMPK and acetyl-CoA carboxylase (ACC), its downstream target. An important decrease of total AMPK (55%), total ACC (57%) and phosphorylated ACC (61%) was present in doxorubicin treated males. Importantly, this was not the case for the treated females (Figure 4A). As AMPK is a central regulator of both lipid and glucose metabolism, we analyzed gene expression involved in glucose and fatty acid utilization in the left ventricle. There was an important decrease in the expression of the glucose transporter Glut4 and of hexokinase 2 (HK2), which significantly correlated to total AMPK (R=0.55, p=0.01 and R=0.70, p=0.003, respectively), while pyruvate dehydrogenase kinase 4 (Pdk4) expression increased in doxorubicin treated males (Figure 4B), pointing to a decrease in carbohydrate metabolism. For the treated females, no or a small reduction of the glucose metabolism genes was observed. While total lactate dehydrogenase (LDH) activity was preserved (Figure 4D), a significant decrease of H and increase of M LDH isoform were observed only in males treated with doxorubicin (Figure S3A). Regarding lipid metabolism, no sex difference was noticed in the decrease of carnitine palmitoyltransferase 1b (Cpt1b) and medium-chain acyl-CoA dehydrogenase (Mcad) induced by doxorubicin treatment (Figure 4C). These results showed that doxorubicin induced a deregulation of cardiac energy homeostasis in males while normal energy metabolism is maintained in females. Cardiac energy homeostasis appeared as one of the sites of sex differences as evidenced by significant statistical interactions between sex and treatment effects (Table S1).
Doxorubicin severely impairs mitochondrial biogenesis in males

No significant changes were observed in citrate synthase (CS), cytochrome oxidase (COX), and total and mitochondrial creatine kinase (CK) activities in both sexes after 7 weeks of doxorubicin treatment (Figure 4D and Figure S3B). Nevertheless, a trend for COX and CK activities was noticed for treated males (Figure 4D). We analyzed protein and mRNA levels of the transcriptional coactivator peroxisome proliferator-activated receptor-coactivator1 (Pgc-1α), master regulators of mitochondrial biogenesis, and their downstream targets. No modification of PGC-1α protein level was observed, however, mRNA levels of Pgc-1α and its homologue Pgc-1β were strongly reduced only in doxorubicin-treated males (Figure 5A and 5B). Downstream genes involved in mitochondria biogenesis were highly down-regulated in treated-males while the deregulation was moderate or even absent (like transcription factor A mitochondrial Tfam) for the treated females (Figure 5B). No effect of doxorubicin was observed in females for COX subunit IV isoform1 (Cox4) and optic atrophy 1 (Opa1) gene expression, while there was a strong decrease in treated males. For both sexes, adenine nucleotide translocator 1 (Ant1), voltage-dependent anion channel 1 (Vdac1), Cs and mitofusin2 (Mfn2) were strongly decreased (Figure 6A). However, we did not observe variation in the level of the electron transport chain proteins and other mitochondrial proteins content (Figure S3C). As AMPK and more recently PGC-1α/β have been shown to regulate cardiolipin homeostasis, the cardiolipin content was measured.15, 16 A strong reduction was revealed only in doxorubicin-treated males (Figure 6B). Mitochondrial biogenesis and cardiolipin environment emerged as other key sites of doxorubicin related sex differences as there is a significant statistical interaction between sex and treatment effects (Table S1). Taken together, these results show that mitochondrial biogenesis is a specific target of doxorubicin in males.
Early cardiac dysfunction in males is linked to altered mitochondrial function

To unravel the onset of cardiac dysfunction, we shortened the experiments to 4 weeks of doxorubicin treatment. Once again, treated females had minor anatomical and no cardiac alterations while treated males developed major signs of cardiomyopathy with cardiac atrophy, reduced LVEF, increased levels of Anf and Il6 (Table S2, Figure 7A). Surprisingly, Pgc-1α mRNA level was similar in all groups whereas Pgc-1β was reduced only in doxorubicin-treated males (Figure 7B). To investigate mitochondrial physiology, the activity of the respiratory chain and the ability of mitochondria to oxidize different substrates were studied in left ventricular permeabilized fibers at 4 weeks by measuring oxygen consumption rates (Figure 7C). No alteration was observed in treated females. In contrast, respiration rate with malate+palmitoyl-CoA+carnitine was reduced in treated males from 13.6±1.1 to 10.7±0.5 μmol O₂/min/g dw, p<0.04 (Figure 7C). Similarly, the maximal oxygen consumption rate in the presence of mitochondrial substrate’s mixture (malate+palmitoyl-CoA+carnitine, +pyruvate, +glutamate and succinate), was reduced only in doxorubicin-treated males from 24.5±1.5 to 19.6±1.0 μmol O₂/min/g dw (p<0.05). We analyzed mitochondrial DNA content normalized to nuclear DNA (Figure 7D). Again, we observed a decrease for doxorubicin-treated males only. Finally, global protein acetylation in the left ventricles (Figure 7E), was reduced only for doxorubicin-treated males (Male NT 0.21±0.02 acetylation level normalized to coomassie vs 0.13±0.01 Male DOXO (p=0.003); Female NT 0.17±0.02 vs 0.18±0.01 Female DOXO). Altogether, these results suggest that mitochondrial dysfunction participates in early cardiotoxicity in males and in sex-related differences as shown by the interaction between sex and doxorubicin for mitochondrial function (Figure 7F).
Discussion

In the present study, we explored sexual dimorphism of doxorubicin cardiotoxicity and energetic and signaling pathways that could be involved in these differences. Two clinically relevant cumulative doses of doxorubicin, either 14 mg/kg after 7 injections or 8 mg/kg after 4 injections were administrated to investigate sex differences in the cardiotoxicity of doxorubicin. Doxorubicin treatment resulted in sex differences characterized in males by 1) important weight loss and decrease in survival rate 2) strong alterations of myocardial function 3) decrease in energy signaling pathways 4) down regulation of mitochondrial biogenesis 5) decrease in cardiolipin content 6) decrease in mitochondrial DNA content 7) and alteration of mitochondrial respiration. No sex differences were found for the oxidative stress response or for death markers, whereas mitochondrial dysfunction and mitochondrial protein expression were associated with early cardiotoxicity in males.

Several clinical studies and animal models indicate that dose-dependent doxorubicin cardiotoxicity increases with age. Colombo et al showed that chemotherapy with anthracycline results in higher toxicity in 24-month-old compared to 6 month-old-rats. 3-month-old male rats are far more sensitive to doxorubicin than age-matched females. The mortality rate was as high as 50% in males after 7 weeks of treatment, while all females survived. Hepatomegaly appeared slightly more important in females however liver aspect was identical in both sexes. Similarly, liver weight increase, probably attributable to cellular proliferation, was higher in females than males. The authors showed that the liver oxidative defence system was far less susceptible to in vivo doxorubicin treatment in female than male rats. As expected, doxorubicin treatment induced dyslipidemia in both sexes. However, it was mild at four weeks in females and increased thereafter while it was already high at 4 weeks in males.
The present results clearly evidence early signs of cardiac dysfunction and heart failure only in males with cardiac atrophy, which is a hallmark of anthracycline toxicity. Females had an almost preserved LVEF, while males had a severe decrease in LVEF combined with pulmonary edema, ascites, structural myocardial alterations including myolysis and fibrosis and high levels of Il-6, Anf and Mcip1 mRNA, which have all been correlated with the severity of left ventricular dysfunction. Thus, females are better protected from doxorubicin-induced heart failure.

Oxidative stress leading to increased cell death has been widely reported as a key mechanism in the development of doxorubicin toxicity, although anti-oxidant therapy in humans was not able to prevent heart failure induced by anthracyclines. Females may produce less mitochondrial free radicals and have a higher content of antioxidant enzymes than males, these differences having been suggested as a mechanism promoting longevity and preventing cardiovascular diseases. Estrogen receptors and their signaling cascades have been implicated in the control of anti-oxidant enzyme expression. However, in our model, doxorubicin did not induce obvious global protein oxidation or sex difference in this parameter. Moreover, level of Gpx1 was unchanged. Nevertheless, decreased levels of Sod2 and Catalase were more important in males after doxorubicin. Recent data reinforced the role of mitochondrial iron accumulation and cellular ROS in doxorubicin cardiotoxicity. The redox balance is certainly altered after doxorubicin treatment. However, the level and importance of oxidative stress is surely time- species- and concentration-dependent leading to different results according to the model of doxorubicin treatment. Cardiomyocyte loss through various cell death signaling pathways linked to oxidative stress and/or DNA damage and/or mitochondrial alterations, has been described in doxorubicin cardiotoxicity. However, depending on in vitro and in vivo analysis, the results were not always consistent. In the present study, no sex difference in cell death was observed in
vivo and in vitro after doxorubicin. These results match those of the gene expression analysis of cell death markers that did not change in both sexes after doxorubicin treatment. Absence of sex-difference in the apoptosis signaling transduction cascade was already reported in vivo after a single i.p. injection of 10mg/kg of doxorubicin, although this study did not describe cardiac damage in both sexes. Thus, neither oxidative stress nor cell death seems to be a strong determinant of sexual dimorphism in doxorubicin-cardiotoxicity.

Mitochondrial dysfunction has been recently highlighted as a major determinant of anthracycline cardiotoxicity. Moreover, energy depletion and mitochondrial dysfunction are well known characteristics of the failing heart. Following transverse aortic constriction (TAC) in mice, better preserved cardiac function in females is associated with fewer alteration of mitochondrial function and biogenesis as well as fatty acid oxidation. This was explained by the transcriptional control of Pgc-1α gene expression by estradiol. Indeed mitochondrial function and biogenesis are under the control of estrogens. Moreover, PGC-1α and PGC-1β modulate estrogen receptor alpha transcriptional activity. It thus appears that there is a tight relation between PGC-1s, mitochondrial functions and female sex hormones. Indeed, doxorubicin treatment also altered mitochondria more severely in males, as evidenced by the down-regulation of gene expression of mitochondrial biogenesis (Pgc-1s and their downstream transcription cascade), mitochondrial function (Cox, Cs, PTP members) and mitochondrial dynamic (Mfn2 and Opa1), decreased mitochondrial respiration and mitochondrial DNA content. PGC-1s have been characterized as orchestrators for mitochondria biogenesis, substrate preference and antioxidant defenses. PGC-1α and its targets are down-regulated in different experimental models, of heart failure as well as in patients. The same was found in daunorubicin-treated male rabbits. In the present study, PGC-1β appeared a relevant target of doxorubicin in males since its down-regulation is also observed at the onset of cardiac toxicity. PGC-1β discovered a decade ago, is expressed
predominantly in skeletal muscle, heart, brain and brown adipose tissue.\textsuperscript{42, 43} In pressure overload hypertrophy, PGC-1\textbeta was described as an important factor in maintaining mitochondrial function by preserving glucose metabolism\textsuperscript{44} and could be a key parameter in sex-related doxorubicin cardiotoxicity differences. The pivotal role played by mitochondrial dysfunction in doxorubicin cardiotoxicity is furthermore revealed by the fact that activation of mitochondrial function or biogenesis by various agents like frataxin overexpression, exercise training or resveratrol, are sufficient to protect against doxorubicin cardiotoxicity and cardiac dysfunction.\textsuperscript{45, 46} In the present study, doxorubicin cardiotoxicity was observed only in males and was accompanied by early mitochondrial dysfunction again emphasizing the role of mitochondrial dysfunction in doxorubicin cardiotoxicity and further suggesting a possible role in sex-differences.

Finally, AMPK, a kinase activated by the low energy status, has been reported to be a cardiac target of doxorubicin.\textsuperscript{12} Activation of AMPK via up-regulation of adiponectin, a hormone secreted by adipose tissue, or via treatment with metformin, an antidiabetic drug, improved the energy state, mitochondrial function and cardiac contractility altered by doxorubicin.\textsuperscript{47, 48} Once activated, AMPK stimulates ATP production and inhibits ATP consuming processes and is a key factor for the regulation of mitochondrial biogenesis.\textsuperscript{49} In treated-males but not females, AMPK, its important target ACC, as well as gene expression of proteins involved in glucose and fatty acid utilization were down-regulated. Importantly, a sex difference was observed in glucose metabolism as evidenced by Pdk4 and M-LDH increase, converging towards impairment of oxidative metabolism in males. AMPK may have a potential cardioprotective role in females by limiting glucose metabolism alteration and maintaining normal mitochondrial biogenesis. Interestingly, a significant correlation was found between Pgc-1\textbeta and total AMPK (R=0.62, p=0.01). Moreover these two pathways also control cardiolipin homeostasis. Interestingly, several studies have shown that doxorubicin binds with
high affinity to cardiolipin.\textsuperscript{50} We previously showed that AMPK regulates cardiolipin synthesis and remodeling.\textsuperscript{15} Recently PGC-1α/β were also described as cardiolipin synthesis modulators.\textsuperscript{16} These data are in accordance with our results showing that doxorubicin treated males displayed almost normal \textit{in vitro} mitochondrial protein content and activity while \textit{in situ} oxidative capacity are reduced probably due to altered cardiolipin environment of the respiratory chain.

At present, clinical studies aimed at analyzing the difference between sexes with respect to cardiotoxicity caused by anticancer therapy are still sparse. This study has unraveled a significant sexual dimorphism in the sensitivity to doxorubicin in rats. Mitochondrial dysfunction and altered energy signaling pathways together with altered cardiolipin homeostasis appear to play a pivotal role in doxorubicin cardiotoxicity and sex differences (Figure S4). A cause-and-effect relationship between altered AMPK activity and sex differences in cardiotoxicity as well as the role of sex hormones remain to be established. Further investigations are needed to decipher the upstream mechanisms of energy metabolism remodeling in males in order to define novel therapeutic targets to prevent anthracycline cardiotoxicity.

\textbf{Acknowledgements}

We thank Valérie Domergue and Pauline Robert (Animal facility of IFR141-IPSIT) for their help with the animals, Claudine Deloménie (Transcriptomic platform of IFR141-IPSIT) for her expertise in molecular biology, Eric Jacquet and Prishila Ponien (Platform Imagif, CNRS Gif/Yvette) for TLDA experiments. We thank Nicolas Raymond for his excellent histological work and the Microscopy Facility of the Centre Chirurgical Marie Lannelongue. We thank Antoinette Lemoine, AP-HP, Hôpital Paul Brousse for help with doxorubicin. We thank Matthieu Gutmann, Christophe Lemaire, Eric Morel, Pierre Chaminade, Jean-Louis Paul,
Vincent Algalarrondo (Université de Paris-Sud) for helpful discussion and Rodolphe Fischmeister for continuous support. We finally thank Jesse Schloegel for careful reading of the manuscript.

**Sources of Funding**

Our laboratory is a member of the Laboratory of Excellence LERMIT and is supported by a grant from “Fondation pour la Recherche Médicale”, université Paris-Sud (ERM) and CORDDIM and MALINF-DIM Ile de France. MM is recipient of a fellowship from the “Fondation ARC”. RVC is senior scientist at CNRS.

**Disclosures**

None.

**References**


Table. Anatomical characteristics of male and female rats at 7 weeks of doxorubicin treatment

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<td>7 weeks of treatment</td>
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<td>358 ± 24*</td>
<td>295 ± 9</td>
<td>264 ± 10*</td>
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Mean ± SD is presented. * is for p<0.05 for the effect of doxorubicin treatment.
Figure Legends

Figure 1. Doxorubicin treatment severely decreases survival rate, promotes body wasting and triggers Anf, Il-6 and Mcip1 gene expression in males.

A. Survival analysis in doxorubicin males (n=18 for the first 4 weeks and n=8 after) and females (n=16 for the first 4 weeks and n=6 after). B. Important weight loss from the third week of doxorubicin treatment for the doxorubicin treated male group. Only DOXO statistics are presented; statistical significance p<0.01 (**) and p<0.001 (***, $$$); * for NT versus DOXO and $ for FEMALE DOXO versus MALE DOXO. C. Markers of cardiac stress: Anf (atrial natriuretic factor), Mcip1 (myocyte-enriched calcineurin interacting protein 1) and Il6 (interleukin-6) expression. D. Representative of fibrosis analysis by Sirius red staining of sub-equatorial heart section from 7 weeks doxorubicin-treated rat. E. Representative histological analysis by hematoxylin and eosin staining of sub-equatorial heart sections from 7 weeks doxorubicin-treated rats.

Figure 2. Severe cardiomyopathy is present only in doxorubicin treated males.

A. Cardiac function was assessed by echocardiography after 7 weeks of doxorubicin (DOXO) or saline (NT) treatment in male (n=4) and female (n=6) rats. B. Statistical analysis by two-way ANOVA. FS, fractional shortening; LVEF, left ventricular ejection fraction; LVIDs, systolic LV internal diameter; LVIDd, diastolic LVID; IVSd and s, interventricular septal wall thickness in diastole and systole; CO, cardiac output.
Figure 3. Doxorubicin does not change cell death and oxidative stress pathway in a sex-specific manner.

A. Myocyte cell death analysis by TUNEL staining of sub-equatorial heart section from 7 weeks doxorubicin-treated rats. B. Cardiomyocytes from adult male or female rats were treated for 24h with 100μM doxorubicin, stained with calcein-AM and then analyzed by flow cytometry. The top panel is cell size and granularity and bottom panel is calcein-staining analysis (SSC: size scatter and FSC: forward scatter). C. Histogram representation of results from 4 to 8 experiments described in B. D. Gene expression of cell death markers in left ventricles of 7 weeks doxorubicin-treated rats: Tnfa, Bcl2, Beclin1, Chop and Grp78. E. Gene expression of oxidative stress markers in 7 weeks doxorubicin-treated rats: Sod2, Gpx1 and catalase. F. Global carbonylation of ventricle proteins.

Figure 4. AMPK signaling is inhibited by doxorubicin only in males

A. Cardiac total and phosphorylated AMPK (AMP-activated kinase) and its target ACC (acylCoA carboxylase) were unchanged in 7 weeks doxorubicin treated females but were highly down-regulated in treated males. B. Gene expression of glucose metabolism: Glut4, Hk2, Pdk4. C. Gene expression of fatty acid utilization: Cpt1 and Mcad. D. Markers of mitochondrial mass and function: COX, CK, CS and LDH activities.

Figure 5. Mitochondrial biogenesis is strongly weakened in doxorubicin treated males.

A. Left ventricular protein level of PGC-1α after 7 weeks doxorubicin (DOXO) or saline (NT) treated rats. B. Markers of mitochondria biogenesis: mRNA expression of Pgc-1α, Pgc-1β, Erra, Tfam, Ppara and Pparβ/δ.
Figure 6. Mitochondrial markers are more severely altered in males than females.

A. Mitochondrial gene expression analysis in ventricles of 7 weeks doxorubicin-treated rats: Cox1, Cox4, Cs, Ant1, Vdac, Mfn2 and Opa1. B. Cardiolipin content in ventricles of 7 weeks doxorubicin-treated rats.

Figure 7. Cardiac stress and mitochondrial dysfunction are already evidenced at 4 weeks of doxorubicin treatment only in males.

A. Markers of cardiac damage: Anf and Il6 expression after 4 weeks of doxorubicin treatment. B. Pgc-1α and Pgc-1β gene expression analysis at 4 weeks of doxorubicin treatment. C. Mitochondrial substrate utilization assessed by respiration of permeabilized cardiac fibers from 4 weeks doxorubicin-treated rats using cumulative addition of 4 mM malate, 1 mM carnitine and 0.1 mM palmitoyl-CoA, 1 mM pyruvate, 15 mM succinate. D. Mitochondrial DNA content analysis after 4 weeks of doxorubicin treatment. E. Representative global protein acetylation analysis in ventricles of 4 weeks doxorubicin-treated rats. F. Statistical analysis of sex, doxorubicin treatment and interaction between sex and treatment at 7 weeks of treatment.
Figure 1

A

Percent survival

MALE DOXO

FEMALE DOXO

Days

p=0.11

p=0.002

p=0.036

Body weight gain or loss (%)

MALE NT

MALE DOXO

FEMALE NT

FEMALE DOXO

Days

p<0.001

p=0.028

p=0.14

p=0.003

p=0.008

p=0.47

Relative mRNA

Anf

Interleukin 6

Mclp1

MALE NT

MALE DOXO

FEMALE NT

FEMALE DOXO

E

MALE NT

FEMALE NT

MALE DOXO

FEMALE DOXO
Figure 2

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Figure 3

A. MALE NT, FEMALE NT, MALE DOXO, FEMALE DOXO

B. MALE NT, MALE DOXO, FEMALE NT, FEMALE DOXO

C. % viable cells (calcein positive)

D. No significant sex difference

E. No significant sex difference

F. oxyblot, coomassie

No significant sex difference

MALE NT, FEMALE NT, MALE DOXO, FEMALE DOXO
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- p=0.003
- p=0.028
- p=0.51
- p=0.89
- p=0.17
- p=0.002
- p<0.001
- p<0.001
- p=0.002
- p=0.01
- p=0.004
- p=0.56
- p=0.001
- p=0.08
- p=0.001
- p=0.50
- p<0.001
- p=0.033
- p=0.74
Figure 5

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PGC1α

β-actin

B

![Bar graph showing relative mRNA levels for Pgc-1α and Pgc-1β](image)

** p=0.020  p=0.045
*** p=0.002  p=0.026
*** **

![Bar graph showing relative mRNA levels for Err α, Tfam, Ppar α, and Ppar β/δ](image)

** p=0.005  p=0.31
Figure 7

Statistical analysis

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Sexual Dimorphism of Doxorubicin-Mediated Cardiotoxicity: Potential Role of Energy Metabolism Remodeling
Maryline Moulin, Jérôme Piquereau, Philippe Mateo, Dominique Fortin, Catherine Rucker-Martin, Mélanie Gressette, Florence Lefebvre, Milada Gresikova, Audrey Solgadi, Vladimir Veksler, Anne Garnier and Renée Ventura-Clapier

Circ Heart Fail. published online November 24, 2014;
Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

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Supplemental methods

Animal conditioning

A chronic model of doxorubicin\(^1\) was chosen by weekly intravenous injection of 2 mg/kg doxorubicin or saline solution during 7 weeks in 16 male and 12 female adult Wistar rats (Janvier Labs). Doxorubicin treatment started when rats were 11 week-old after a 1-week acclimation period. The animals were housed 3 or 4 per cage in a temperature-controlled room (22°C), with a 12/12 h light/dark cycle, and were provided food and water *ad libitum*.

Echocardiography

Echocardiography was performed at 4 and 7 weeks of doxorubicin treatment using a 12 MHz transducer (Vivid 7, General Electric Healthcare) under 2.5% isoflurane gas anesthesia. Two-dimensional-guided (2D) M-mode echocardiography was used to determine wall thickness and left ventricular chamber diameter during systole and diastole, and contractile parameters such as fractional shortening (FS) or ejection fraction (EF%).

Sacrifices

At the end of the treatments, all rats were anesthetized by intraperitoneal injection of pentobarbital (200 mg/kg). The depth of anesthesia was checked by toe pinch before the start of surgery. The heart was then quickly removed for euthanasia then liver, kidney, spleen, lung and tibia were collected. Part of the hearts was immediately used for functional experiments, and another part was rapidly frozen and kept at −80°C for further investigations.

Mitochondrial function

Oxygen consumption was measured on saponin-permeabilized cardiac fibers.\(^2\)\(^,\)\(^3\) Oxygen consumption was followed in the presence of 2 mM ADP and 4 mM malate, by cumulative additions of substrates: palmitoyl-CoA 0.1 mM plus carnitine 1 mM, pyruvate 1 mM, glutamate 10 mM, succinate 15 mM. Maximal respiration was measured after succinate addition. Rates of respiration are given in μmoles O\(_2\)/min/g dry weight (dw). Three experiments per heart were performed and at least 6 animals per group were used.

Mitochondrial (mtDNA) and nuclear (nDNA) DNA content

Total DNA was extracted from left ventricles. Briefly, tissues were minced in 500μl of buffer 1 (TrisHCl 10 mM, NaCl 10 mM, EDTA 25 mM, SDS 1%, proteinase K 0.4mg/ml, pH 7.5) with cleaned scissors and incubated overnight at 37°C. After addition of 50μl of saturated NaCl solution, the homogenates were centrifuged at 500g for 15 minutes. Isopropanol (500μl) was added to supernatants which were then incubated 45 minutes at -20°C. After a centrifugation at 16000 g for 20 minutes, the pellets were kept, washed with ethanol 70%, resuspended in buffer 2 (TrisHCl 10 mM, EDTA 1 mM, RNAase A 0.033mg/ml, pH 8) and
finally warmed at 70°C. mtDNA content was measured by real-time qPCR using specific primers for a mitochondrial gene (16S, forward primer CTAGAAACCCGAAACCAA, reverse primer CCAGCTATCAACCAAGCTCGT) and a nuclear gene (B2m (beta-2-microglobulin), forward primer TGGTAAAGCAAAGAGGCCTAA, reverse primer AGAAGTGCCACAGGGTGG). The difference between the threshold cycle (Ct) of nuclear gene and mtDNA gene (ΔCt) was used to assess the mtDNA/nDNA ratio, which was calculated using the following formula: 2(2−ΔCt).

Real-time quantitative PCR analysis

Total ventricular RNA was extracted using Trizol reagent (Invitrogen). Two µg of total RNA was used to synthesize cDNA according to the protocol provided with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, France). Real-time PCR was performed using TaqMan Low Density Array (TLDA) technology. TLDA were designed to amplify 48 cDNA for each sample as follows: 43 target genes involved in energy metabolism, mitochondrial function and cell death pathway and 5 housekeeping genes. Quantification was achieved using the ΔΔCt method. The average Ct obtained in non-treated male group was used as a calibrator and ribosomal protein P2 (Rplp2) housekeeping gene was used as the reference for normalization.

Biochemical studies

Frozen tissue samples were weighed, homogenized (Bertin Precellys 24) in ice-cold buffer (50 mg/ml) containing HEPES 5 mM (pH 8.7), EGTA 1 mM, DTT 1mM and 0.1% Triton X-100. Citrate synthase (CS), cytochrome oxidase (COX), lactate dehydrogenase (LDH) and total creatine kinase (CK) activities were determined in homogenized ventricles as previously described. LDH and CK isoenzyme were separated using agarose gel electrophoresis performed at 200 V for 90 minutes. Individual isoenzymes were resolved by incubation of the gels with a coupled enzyme system as previously described.

For Western blot experiments, frozen tissue samples were homogenized (Bertin Precellys 24) in ice cold buffer containing Hepes 50 mM, KCl 50 mM, EDTA 1 mM, beta-glycerophosphate 5 mM, Triton X100 0.1%, orthovanadate 1 mM, DTT 1 mM, NaF 50 mM, NaPPi 5 mM, PMSF 0.2 mM and antiprotease cocktail set. Samples were separated on 8 to 12% polyacrylamide gels and transferred to PVDF membranes for antibody detection. After Ponceau staining, all membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20. Antibodies were used as follows: monoclonal anti-β-actin (Santa Cruz sc-47778), phospho- and total AMPK (Cell Signaling 2531-2532), phospho- and total ACC (Cell Signaling 3661-3676), PGC-1α (Santa cruz sc-13067), CS (BD biosciences 612606), oxyblot (Millipore). Washing steps were performed with PBS-T. After incubating with HRP-coupled secondary antibodies, Western blots were visualized by ECL (Millipore).

Cardiomyocyte cultures

Male and Female Wistar rats (12-16 weeks old) were subjected to anesthesia by intraperitoneal injection of pentobarbital (200 mg/kg) and hearts were rapidly excised. Individual adult rat ventricular myocytes were dissociated by retrograde perfusion of the heart with collagenase as described previously. Freshly isolated cells were plated on laminin-coated culture dishes in minimal essential medium (M4780, Sigma) supplemented with 2.5% fetal bovine serum, penicillin (100units/mL), streptomycin (100µg/mL), and 2% HEPES (pH
7.4) for 1 hour, and switched to serum-free medium overnight before 24h doxorubicin treatment.

Flow cytometry analysis

Adult rat cardiomyocytes were either kept untreated or exposed for 24h to various concentrations of doxorubicin. At the end of the treatment, cells were incubated 5 min with 1 μM acetomethoxyl derivate of calcein (calcein-AM) at 37°C. Then, medium was spun to collect dead cells and cell pellets were resuspended in PBS. Attached cardiomyocytes were gently detached with PBS and both cell fractions were combined prior being analysis by a FACScalibur flow cytometry (BD Biosciences). FL1 filter (for calcein staining) was used to analyze 10,000 cell populations. In viable cells, the non fluorescent calcein-AM is transformed into a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. As dead cells lack active esterases, only viable cells are labeled and counted by flow cytometry. Results are presented as the percentage of cell viability (percentage of calcein positive cells).

Histological assessment

Hearts were fixed in 4% paraformaldehyde, paraffin embedded and serially sectioned (5 µm). Sections were stained with Sirius red, or hematoxylin and eosin or used unstained for terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) which were conducted as described previously. Fibrosis and cell death quantification were performed on 3-4 sections (5-10 fields/section) per animal.

Doxorubicin analysis

Doxorubicin analysis was performed by liquid chromatography-electrospray ionization-tandem mass spectroscopy (Quatro Ultima, Waters) according to. Briefly, frozen tissue samples were homogenized (Bertin Precellys 24) in 5% w/v solvent (60% acetonitrile – 40% 5mM ammonium acetate pH3.5) followed by centrifugation. Daunorubicin was used as internal standard. The mobile phase consisted of 40% acetonitrile and 60% acetic acid and isocratic conditions were used at a flow rate of 250µL/min. A volume of 10 µL was injected onto the column.

Triglyceride and cholesterol quantification

Plasma triglycerides and total cholesterol were determined enzymatically according to manufacturer's instructions using Biomerieux kits and expressed as mmol/L of serum.

Cardiolipin content

Cardiolipin analysis was performed by liquid chromatography using corona-CAD detector. A Folch method was used to extract the lipids from 10 to 20 mg of heart homogenized in PBS. Total lipids were extracted by adding 1.5mL of methanol and 3mL of chloroform to the tissue suspension. After centrifugation at 1000g for 10 min, the lower phase containing total lipids was collected and evaporated to dryness at room temperature under nitrogen gas. The samples were resuspended in 100µL of chloroform per 10 mg of heart and subsequently analyzed. Quantification of cardiolipin was performed on a Dionex U-3000 RSLC system (ThermoFisher Scientific) equipped with a Corona-CAD Ultra (ThermoFisher Scientific).
separation of lipids was performed with a PVA-Sil column (150 x 2.1 mm I.D., 120 A) (YMC Europe GmbH) at 35°C. Chromatographic method was inspired from the method developed by Imbert et al. The flow rate was set at 0.400mL/min and 5µL of sample were injected. The corona-CAD nebulizer was set at 30°C and the nitrogen pressure was set at 5 bar. Standard curve from 0.5 to 0.025mg/mL of cardiolipin sodium salt from bovine heart (98% purity from Sigma-Aldrich) was used.
Table S1

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Table S1: Statistical analysis of sex, doxorubicin treatment and interaction between sex and treatment at 7 weeks of treatment.
Table S2: Anatomical characteristics and echocardiographic data of male and female rats at 4 weeks of doxorubicin treatment

Mean ± SD is presented. Grey background is for p<0.05 for the effect of doxorubicin treatment.
Figure S1

A

B

C

D

Sex and DOXO interaction

DOXO effect

Sex effect

Statistical analysis

Total Fibrosis

Reactive Fibrosis

Perimyocyte fibrosis

Sex effect

DOXO effect

Sex and DOXO interaction

E

F

Statistical analysis

TUNEL Myocytes

TUNEL Total

Sex effect

DOXO effect

Sex and DOXO interaction
Figure S2

A

long exposure
\[ \begin{align*}
\text{H}_2\text{O}_2 & \rightarrow \text{Control} \\
\text{short exposure} & \rightarrow \text{H}_2\text{O}_2 \\
\text{Control} & \rightarrow 
\end{align*} \]

B

\text{oxyblot}

\text{coomassie}

\text{FEMALE}
\begin{align*}
\text{NT} & \rightarrow \\
\text{DOXO} & \rightarrow 
\end{align*}

\text{MALE}
\begin{align*}
\text{NT} & \rightarrow \\
\text{DOXO} & \rightarrow 
\end{align*}
Figure S3

A

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% LDH isoform

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% CK isoform

C

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p=0.06

p=0.050

p=0.77
Figure S4

DOXORUBICIN

AMPK/PGC-1α/β

Cardiolipin content

Mitochondrial biogenesis

Oxidative capacity

Cardiac dysfunction
**Supplemental figure legends**

**Figure S1: Fibrosis analysis**
A. Quantification of total fibrosis from 20 fields for each rat. B. Quantification of reactive fibrosis from 20 fields for each rat. C. Quantification of perimyocyte fibrosis from 20 fields for each rat. D. Fibrosis statistical analysis of sex, doxorubicin treatment and interaction between sex and treatment. E. Cell death analysis by TUNEL staining of sub-equatorial heart section from 7 weeks doxorubicin-treated rats. F. Cell death statistical analysis of sex, doxorubicin treatment and interaction between sex and treatment.

**Figure S2: Oxidative stress analysis**
A. $H_2O_2$ induced protein carbonylation. B. Global carbonylation level of ventricle proteins from 4 weeks doxorubicin-treated rats.

**Figure S3: LDH and CK isoforms analysis and mitochondrial protein analysis**
A. Representative LDH isoform analysis in left ventricles of 7 weeks doxorubicin-treated rats; histogram quantification of LDH isoforms (n=4). B. Representative CK isoform analysis in left ventricles of 7 weeks doxorubicin-treated rats (no dilution or dilution 1/5 was used to avoid saturation); histogram quantification of CK isoforms (n=4). C. Mitochondrial protein levels were similar in both sexes of 7 weeks doxorubicin-treated rats.

**Figure S4: Speculative model of sex-specific cardiac dysfunction after doxorubicin treatment.**
Mitochondrial biogenesis, cardiolipin homeostasis and oxidative capacity are specially altered in doxorubicin-treated males. The energetic sensor AMPK is also specifically down-regulated in males.
Supplemental references


