**Universidade Federal de Ciências da Saúde de Porto Alegre**

**Programa de Pós-Graduação em Ciências da Saúde**

**Verônica Bidinotto Brito**

# Avaliação dos Efeitos Intergeracionais do Exercício Físico e da Suplementação com Resveratrol Durante a Gestação Sobre a Toxicidade da Doxorubicina em Cardiomiócitos de Ratos Neonatos

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**UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE**

**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE**

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Tese submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor em Ciências da Saúde.

Orientadora: Profª. Drª. Jenifer Saffi

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*“É melhor tentar e falhar, que se preocupar e ver a vida passar. É melhor tentar, ainda que em vão, que se sentar fazendo nada até o final. Eu prefiro na chuva caminhar, que em dias tristes em casa me esconder. Prefiro ser feliz, embora louco, que em conformidade viver.”*

*(Martin Luther King)*

**RESUMO**

O antineoplásico Doxorubicina (DOX) possui uso limitado devido à cardiotoxicidade dose-dependente, principalmente relacionada ao estresse oxidativo gerado. Em modelos experimentais, o exercício ou a suplementação com resveratrol durante o tratamento com a DOX tem demonstrado efeitos cardioprotetores importantes. Além disso, tem sido demonstrado que o exercício e compostos bioativos derivados da dieta são capazes de modular parâmetros epigenéticos. O objetivo desse trabalho foi investigar os efeitos intergeracionais do exercício e da suplementação com resveratrol sobre a toxicidade da DOX nos cardiomiócitos da ninhada. Além disso, uma possível herança de cardioproteção foi investigada. Para tal, ratas prenhas foram alocadas em 3 grupos (controle, exercício ou resveratrol), e receberam os tratamentos durante o período gestacional. Após o nascimento dos neonatos, seus corações foram utilizados para a obtenção da cultura de cardiomiócitos que foi tratada com DOX para as análises de: viabilidade celular, apoptose e necrose; produção de espécies reativas de oxigênio (ERO); dano ao DNA; perfil antioxidante; e expressão proteica da sirtuína6 (Sirt6) e catalase (CAT). Os resultados demonstram que o exercício realizado durante o período gestacional aumenta a viabilidade dos cardiomiócitos dos neonatos, diminuindo a morte por apoptose e necrose induzida pela DOX, resultados correlacionados com o decréscimo na produção de ERO e aumento nas defesas antioxidantes. O exercício também protegeu os cardiomiócitos do dano ao DNA, reduzindo as quebras por danos oxidativos. Esses resultados foram semelhantes aos observados quando as genitoras foram suplementadas com resveratrol durante a gestação. Particularmente, o exercício induziu um aumento significativo na expressão protéica da Sirt6 e CAT nos cardiomiócitos da ninhada, efeito modestamente observado nos cardiomiócitos da ninhada do grupo resveratrol. Conclui-se que o exercício ou a suplementação com resveratrol durante a gestação protege o coração da ninhada contra a toxicidade induzida pela DOX, possivelmente por herança de cardioproteção conferida pela modulação do estresse oxidativo com o aumento do perfil antioxidante, bem como pela modulação da integridade do DNA via Sirt6 no coração do neonato.

***Palavras-Chave:*** *doxorubicina; gestação; exercício; resveratrol; estresse oxidativo; Sirt6; dano no DNA.*

**ABSTRACT**

The antineoplastic Doxorubicin (DOX) has limited use due the dose-dependent cardiotoxicity, mainly related to the oxidative stress production. In experimental models of DOX treatment the exercise or supplementation with resveratrol has demonstrated important cardioprotective effects. Also, it has been demonstrated that exercise and bioactive compounds from diet are able to modulate epigenetic parameters. This work aimed to investigate the intergenerational effects of exercise, as well as resveratrol supplementation, on DOX-induced toxicity in cardiomyocytes of the progeny. Moreover, the possible cardioprotective inheritance was examined. For this purpose, pregnant rats were allocated in 3 groups (control, exercise or resveratrol), and pre-treated during gestational days. After born of pups, the hearts were used to obtain the culture of cardiomyocytes that was treated with DOX for analyses of: cell viability, apoptosis and necrosis; reactive oxygen species (ROS) production; DNA damage; antioxidant profile; and sirtuin6 (Sirt6) and catalase (CAT) protein expression. The results demonstrate that the exercise during pregnancy induces an increase in neonatal cardiomyocytes viability, decrease in DOX-induced apoptotic and necrotic death, which was correlated to the decrease in ROS production and increase in antioxidant defenses. Exercise also protected neonatal cardiomyocytes from DOX-induced DNA damage, demonstrating a reduction in the oxidative DNA breaks. Similar results were observed when the mothers were supplemented with resveratrol during pregnancy. Particularly, the exercise induced a significant increase in protein expression of Sirt6 and CAT in cardiomyocytes of the progeny, which was weakly observed in cardiomyocytes of the progeny of the resveratrol group. These results demonstrate that exercise, as well as resveratrol supplementation performed by mothers protect the heart of progeny against DOX-induced toxicity, probably by cardioprotective inheritance awarded by the modulation of oxidative stress due an increase in antioxidant profile, as well as the modulation of DNA integrity via Sirt6 in the neonatal heart.

***Key-Words:*** *Doxorubicin; pregnancy; exercise; resveratrol; oxidative stress; Sirt6; DNA damage.*

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## LISTA DE ABREVIATURAS

BRCA1 – *Breast cancer 1*

CAT – Catalase

c-JUN – *Transcription factor AP-1*

CtIP – C*arboxy-terminal binding protein-interacting protein*

DNR – Daunorubicina

DOX - Doxorubicina

Endo III – Endonuclease III

eNOS – Óxido nítrico sintase endotelial

ERO – Espécies reativas de oxigênio

Fe – Ferro

$Fe^{2+}$ – Íon ferroso

$Fe^{3+}$ – Íon férrico

Fp – Flavoproteína

FPG – Formamidopirimidino DNA glicosilase

GPx – Glutationa peroxidase

GSH/GSSG – Glutationa reduzida/oxidada

$H_2O_2$ – Peróxido de hidrogênio

HATs – Acetil-transferases de Histonas

HDACs – Desacetilases de Histonas

HIF-1$\alpha$ – *Hypoxia-inducible factor 1-alpha*

$HO^-$ – Ânion hidroxil

$HO^\bullet$ – Radical hidroxila

IAM – Infarto agudo do miocárdio

IC95% – Intervalo de confiança de 95%

ICC – Insuficiência cardíaca congestiva

IRPs – Proteínas reguladoras de ferro

MBD2 – *Methyl-binding domain protein-2*

MCF-7 – *Human breast adenocarcinoma cell line; acronym of Michigan Cancer Foundation-7*

Myc-c – *Myc proto-oncogene protein*

NAD(P) – Nicotinamida adenina dinucleotídeo fosfato

$NAD^+$ – Nicotinamida adenina dinucleotídeo

NF-κB – *Factor nuclear kappa B*

NFκB-p65 – *Nuclear factor NF-kappa-B p65 subunit*

$O_2^{\bullet-}$ – Ânion radical superóxido

PARP-1 – *Poly-ADP-ribose polymerase1*

PGC-1α - *Peroxisome proliferator-activated receptor gamma coactivator 1-alpha*

ROS – Reactive oxygen species

RR – Risco relativo

SFR – *Serum-response fator*

-SH – Grupos sulfidrílicos

Sirt6 – Sirtuína6

SNC – Sistema Nervoso Central

SOD – Superóxido dismutase

**SUMÁRIO**

# 1. INTRODUÇÃO

## 1.1. CÂNCER E QUIMIOTERAPIA

### 1.1.1. Aspectos gerais

Câncer é um termo que define coletivamente um grupo de doenças nas quais células anormais perdem o controle do mecanismo de divisão celular, originando um tumor primário, com células que podem disseminar-se e invadir tecidos adjacentes; bem como tecidos de outras regiões do corpo, o que fazem através do sangue e sistema linfático (NCI, 2015). Permanece como uma das doenças de tratamento mais difícil, responsável por aproximadamente 190 mil óbitos por ano no Brasil (INCA, 2016), sendo a segunda principal causa de morte no país. Sua incidência tem aumentado devido ao envelhecimento da população, especialmente em países desenvolvidos, onde a expectativa de vida é maior.

O câncer é normalmente causado por alterações no material genético das células, o que inclui o acúmulo de mutações sucessivas em oncogenes e supressores de genes, resultando num desequilíbrio do ciclo celular (CHAMBERS; GROOM; MACDONALD, 2002; SAMY et al., 2016). A terapia contra a doença está baseada no processo cirúrgico conservador, de remoção tumoral e na radioterapia ou quimioterapia sistêmica. A maioria dos agentes antitumorais em uso clínico contra o câncer tem como objetivo matar as células tumorais malignas através da inibição de mecanismos envolvidos na divisão celular, apresentando ação citostática ou citotóxica (FISI et al., 2016; KUBEČEK et al., 2015). Entretanto, uma maior compreensão da biologia tumoral obtida nas últimas décadas tem provado a necessidade da utilização de drogas antitumorais mais ativas e seletivas.

A quimioterapia contra o câncer certamente não é uma tarefa fácil. Um dos principais problemas associados é a toxicidade não específica de muitas drogas devido à biodistribuição corporal, fazendo com que seja necessária a administração de uma dose elevada para obter uma alta concentração da droga no local do tumor (NYGREN;

LARSSON, 2003). Outro problema encontrado é a aquisição de resistência contra o quimioterápico. Após desenvolver um mecanismo de resistência em reposta à droga, as células podem apresentar resistência cruzada com outras drogas que possuem estrutura ou mecanismo de ação não relacionado, num fenômeno conhecido como resistência multidrogas (KAPSE-MISTRY et al., 2014; KUNJACHAN et al., 2013).

### 1.1.2. Agentes quimioterapêuticos da classe das Antraciclinas

A introdução dos antibióticos antineoplásicos da classe das antraciclinas no tratamento quimioterapêutico de tumores malignos representou um grande marco na medicina. O poderoso efeito antitumoral das antraciclinas ficou particularmente evidente na oncologia pediátrica com o aumento da taxa de sobrevida em cinco anos que passou de 30% na década de 60 para aproximadamente 70-80% no ano de 2006 (JEMAL et al., 2006).

As primeiras drogas da família das antraciclinas tiveram origem em 1950 quando da identificação da daunorubicina (DNR) a partir da *Streptomyces peucetius*, uma espécie de actinobactéria (DI MARCO; CASSINELLI; ARCAMONE, 1981). Em 1960 foi demonstrado que a DNR era bastante efetiva no tratamento de leucemia linfoblástica e mieloblástica aguda e linfomas (TAN et al., 1967). Ainda nessa década foi identificado um derivado da DNR – a 14-hidroxidaunomicina ou Adriamicina, que mais tarde passou a ser chamada doxorubicina (DOX) – a qual apresentou atividade antitumoral mais ampla incluindo um grande número de tumores sólidos e hematológicos (ARCAMONE et al., 1969; DI MARCO; GAETANI; SCARPINATO, 1969). A DOX e a DNR são moléculas de açúcar e aglicônicas (Figura 1). A estrutura aglicona consiste de um anel tetracíclico com grupos quinona-hidroquinona adjacentes nos anéis C e B, um substituinte metoxil no C4 do anel D, e uma cadeia curta no C9 com um grupo carbonil no C13. O açúcar, chamado de daunosamina, está ligado por uma ligação glicosídica ao C7 do anel A e consiste de uma molécula de 3-amino-2,3,6-tridesoxi-L-fucosil. A única diferença entre a DOX e a DNR é que um lado da cadeia de DOX termina com um álcool primário, enquanto que a DNR termina com um metil; diferença que tem

grande importância e consequências no espectro de atividades da DOX e da DNR (MINOTTI et al., 2004).

Desde as investigações iniciais, as antraciclinas continuam sendo frequentemente utilizadas na prática clínica. Particularmente a DOX permanece como componente importante de muitos protocolos quimioterapêuticos pela atuação sobre uma grande variedade de tumores sólidos (YU et al., 2015) e hematológicos, incluindo leucemias (SZWED et al., 2016), linfomas (STRAUS et al., 2011), câncer de mama (ANDERS et al., 2013), pulmão (AMREDDY et al., 2015), mieloma múltiplo (CHERIYATH et al., 2011) e sarcomas (CHOY et al., 2015), dentre outros.

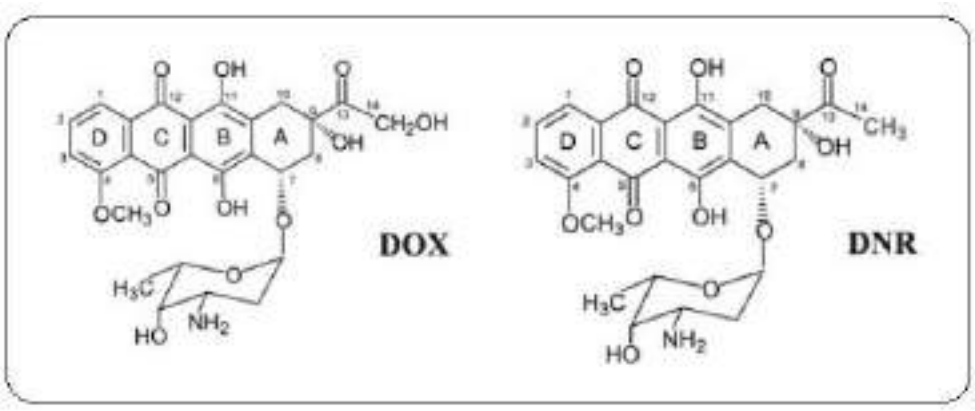


Figura 1: Estrutura química das antraciclinas DOX e DNR. (Reproduzido a partir de Giorgio Minotti et. al. Pharmacol Rev 56: 185–229, 2004).

### 1.1.3. Cardiotoxicidade por Doxorubicina: prevalência, patogênese e prevenção

#### 1.1.3.1. Prevalência

A terapia contra o câncer tem obtido avanços significativos tanto no tratamento de tumores sólidos quanto de doenças hematológicas permitindo que muitos pacientes obtenham a cura. As antraciclinas estão entre os quimioterápicos de uso mais frequente em oncologia, entretanto, como qualquer outro agente antitumoral, seu uso não está livre de complicações, onde o desenvolvimento de resistência em células tumorais e a toxicidade não específica em tecidos saudáveis apresenta uma incidência elevada,

destacando-se a toxicidade cardíaca, desenvolvimento de cardiomiopatia e ICC (insuficiência cardíaca congestiva) como efeitos colaterais mais comuns.

Estudos pré-clínicos em animais falharam na detecção, e somente uma década após a sua descoberta, em finais da década de 70, os primeiros estudos clínicos retrospectivos foram publicados comprovando a presença de alterações cardíacas diretamente relacionadas às repetidas administrações de DOX e DNR; ao mesmo tempo em que foram esses estudos os primeiros a estabelecerem a relação entre a dose cumulativa recebida de antraciclina e o fator de risco para cardiotoxicidade (VON HOFF et al., 1977, 1979).

Apesar da dificuldade de realização de estudos epidemiológicos, há hoje consenso de que o grande determinante para a falência cardíaca após o tratamento com DOX é sua dose cumulativa, tendo em vista o aumento agudo na prevalência de morte cardíaca a partir da dose cumulativa de DOX de 550 mg/m$^2$ de área corporal (GREEN et al., 2001; VILLANI; MEAZZA; MATERAZZO, 2006). Entretanto, em estudos retrospectivos de ensaios clínicos onde a DOX foi utilizada no tratamento do câncer de mama ou de pulmão de pequenas células, foi demonstrado um risco aumentado de cardiotoxicidade em doses de DOX ≤ 300 mg/m$^2$ as quais eram previamente consideradas seguras e improváveis de causar disfunção ventricular esquerda (SWAIN; WHALEY; EWER, 2003) (Tabela 1). Além disso, pacientes que haviam recebido apenas 240 mg/m$^2$ de DOX apresentaram alterações histopatológicas em biópsia do endomiocárdio (BILLINGHAM et al., 1978; BRISTOW et al., 1978a).

Tabela 1: Risco de ICC relacionada à dose cumulativa de DOX administrada.

| **Dose cumulativa (mg/m$^2$)** | **Pacientes com ICC (%)** |
|---|---|
| 150 | 0,2 |
| 300 | 1,6 |
| 450 | 3,3 |
| 600 | 8,7 |

(Dados obtidos em Swain et. al. Cancer 97: 2869-2879, 2003).

A cardiotoxicidade é progressiva e pode ocorrer em vários estágios do tratamento. A cardiotoxicidade “aguda” ocorre durante a administração da DOX ou ao seu término, particularmente quando a droga é administrada na forma em *bolus* ou sob infusão intravenosa rápida, tendo como desfechos típicos vasodilatação, hipotensão e alterações transitórias no fluxo cardíaco (FERRANS et al., 1997; LIPSHULTZ et al., 2012). A toxicidade “subcrônica” é bastante incomum e manifesta-se na forma de síndromes coronarianas com pericardite ou miocardite 1-3 dias após o tratamento, e foi percebida em triagens iniciais onde foram utilizadas doses elevadas de DOX (BRISTOW et al., 1978b; HALE; LEWIS, 1994). O terceiro tipo, a cardiotoxicidade “crônica precoce” desenvolve-se tardiamente no curso do tratamento, ou ainda semanas ou meses após o fim da quimioterapia. Apresenta como características clínicas a cardiomiopatia dilatada, com desenvolvimento subsequente de disfunção ventricular esquerda e ICC (KOCABAŞ et al., 2014). O último subtipo inclui a cardiotoxicidade “crônica tardia” a qual foi descrita inicialmente no início dos anos 90 entre os sobreviventes de câncer na infância (GOORIN et al., 1990; LIPSHULTZ et al., 1991; STEINHERZ; STEINHERZ, 1991), caracterizando a manifestação da cardiotoxicidade por antraciclinas décadas após o fim do tratamento quimioterapêutico.

A cardiotoxicidade crônica tardia é particularmente relevante nos casos de sobreviventes adultos de tumores pediátricos, onde até 65% dos sobreviventes de tumores malignos na infância tratados com DOX/antraciclinas podem apresentar evidências ecocardiográficas de alterações na função contrátil do ventrículo esquerdo (BERGLER-KLEIN et al., 2016; GRENIER; LIPSHULTZ, 1998; LIPSHULTZ, 2006; LIPSHULTZ et al., 1991; VAN DALEN; CARON; KREMER, 2007). Em um estudo de seguimento de 14358 indivíduos com sobrevida após 5 anos, os quais tiveram câncer na infância, foi observado que o uso de DOX em dose < 250 mg/m$^2$ tinha associação com o risco de desenvolver ICC 2,4 vezes maior comparado aos sobreviventes os quais não receberam a droga. Além disso, o risco de desenvolver ICC aumentou para 5,2 vezes com o uso de DOX em dose > 250 mg/m$^2$ (MULROONEY et al., 2009).

Enquanto a cardiotoxicidade aguda não constitui uma complicação clínica maior e normalmente tem resolução rápida e espontânea ao final da infusão, os subtipos de cardiotoxicidade crônica são sérios e clinicamente relevantes, uma vez que

representam grande percentual de morbi-mortalidade e requerem longo tempo de tratamento. Além disso, o aparecimento de complicações cardíacas pode determinar interrupção do tratamento quimioterapêutico e comprometer a cura ou o adequado controle do câncer (SCHLITT et al., 2014; YEH; BICKFORD, 2009).

Ainda, a ocorrência da disfunção ventricular sistólica e diastólica assintomática ou sintomática varia entre 5% e 30%, sendo mais frequente em pacientes que apresentam fatores de risco como: extremos de idade, disfunção ventricular prévia, hipertensão arterial, diabetes, uso de associação de quimioterápicos, radioterapia mediastinal e susceptibilidade genética (CARAM et al., 2015; SENGUPTA et al., 2008; SINGAL; ILISKOVIC, 1998). Salientando que os efeitos cardiotóxicos clássicos das antraciclinas são cumulativos e têm relação com a dose, a velocidade de infusão, a associação de drogas e as insuficiências hepática e renal. Entretanto, qualquer quimioterápico tem potencial para causar toxicidade.

#### 1.1.3.2. Patogênese

Antes de se abordar os mecanismos de ação da DOX é importante mencionar a concentração atingida pela droga na circulação e sustentada pelos pacientes sob tratamento. No esquema de administração em *bolus* variando entre 15 e 90 mg/m$^2$, a concentração plasmática inicial detectada foi de aproximadamente 5 μM (BRENNER et al., 1985; GREENE et al., 1983), enquanto que a concentração mais baixa observada foi de 0,3 μM (KOKENBERG et al., 1988). No entanto, geralmente a concentração plasmática inicial fica na faixa de 1-2 μM (BENJAMIN; RIGGS; BACHUR, 1993; CAMAGGI et al., 1988; CREASEY et al., 1976; MULLER et al., 1993; SPETH et al., 1987a, 1987b). Além disso, a concentração plasmática decai rapidamente, atingindo a faixa de 25-250 ηM dentro de 1 hora – concentração similar àquela atingida e mantida pela infusão contínua (GREENE et al., 1983; KOKENBERG et al., 1988; MULLER et al., 1993; SPETH et al., 1987a, 1987b). Dessa forma, os estudos envolvendo células intactas devem utilizar concentrações de DOX abaixo de 1 ou 2 μM para poderem fornecer informação adequada sobre os potenciais mecanismos de ação do quimioterápico associados ao seu uso clínico (GEWIRTZ, 1999).

Inúmeros mecanismos têm sido propostos para a morte celular induzida pela DOX em doses clínicas relevantes, onde se destacam:

#### *1.1.3.2.1. Veneno de Topoisomerase II*

As topoisomerases são enzimas que regulam a topologia do DNA e facilitam os processos de replicação e transcrição do DNA. Muitas drogas anticâncer, tais como as campotecinas, o etoposido e as quinolonas têm como alvo a inibição de topoisomerases com vistas a matar a célula tumoral (POMMIER et al., 2010). Esse modelo é visto com cautela para a DOX, onde o quimioterápico causaria inibição da Toposisomerase II, quebras duplas no DNA e consequente morte celular, mas somente em elevadas doses clínicas da droga (GEWIRTZ, 1999; MINOTTI et al., 2004). Entretanto, existem outros exemplos nos quais a morte celular causada pela DOX é independente da Topoisomerase II (PANG et al., 2013; ROCHA et al., 2016; SWIFT et al., 2006).

#### *1.1.3.2.2. Formação de adutos com o DNA*

A DOX pode ter ação de agente intercalante do DNA. Nessas situações, forma adutos com o DNA com subsequente ativação de respostas ao dano ao DNA, as quais induzem morte celular independente de Toposisomerase II (CHAIRES; HERRERA; WARING, 1990; FORREST et al., 2012; SAFFI et al., 2010; SWIFT et al., 2006; VAVROVA et al., 2013; ZHANG et al., 2012). A formação de adutos DOX-DNA ocorre em doses clínicas relevantes de DOX, possivelmente ainda durante a quimioterapia (COLDWELL et al., 2008). A interação entre DOX e DNA pode ser estabilizada por uma ligação covalente mediada por um formaldeído celular, formado por reações de radicais livres com fontes carbonadas (TAATJES et al., 1996, 1997). Assim, a interação se estabelece com a formação de uma ligação covalente entre a DOX e uma guanina de uma fita do DNA, mediada pelo formaldeído; e uma ponte de hidrogênio entre a DOX e a outra guanina da fita oposta (Figura 2).

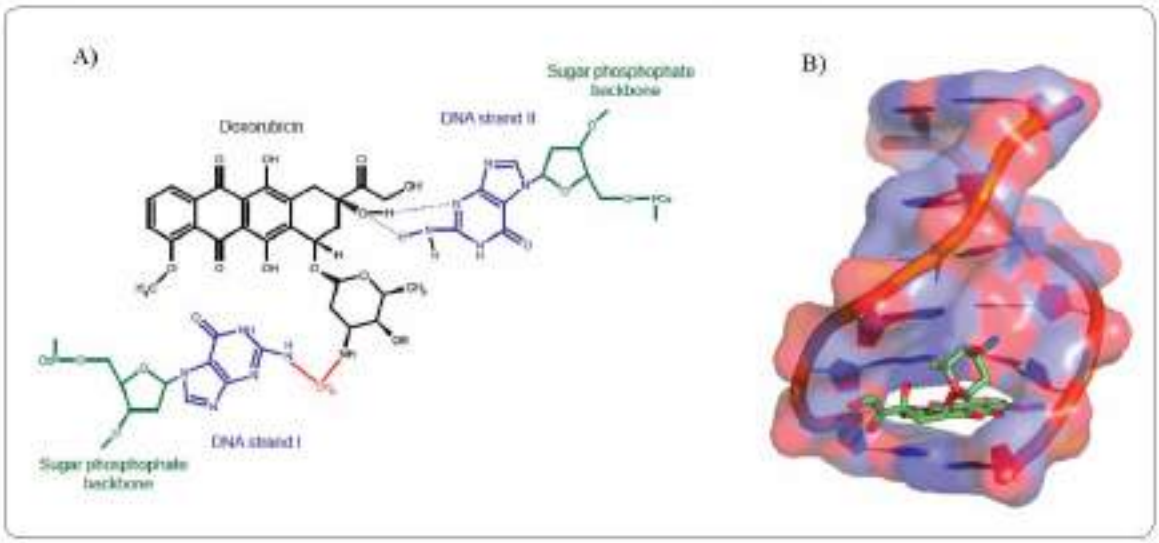


Figura 2: Ligações formadas (A) e estrutura da intercalação entre a DOX e o DNA (B). (Reproduzido a partir de Yang et. al. Biochim Biophys Acta 1845: 84–89, 2014).

*1.1.3.2.3. Aumento na produção de ceramida*

Paralelamente a outros mecanismos, o tratamento com DOX aumenta os níveis de ceramida, uma molécula lipídica envolvida em inúmeros processos celulares como parada no ciclo celular, apoptose e senescência (SENCHENKOV; LITVAK; CABOT, 2001). Em linhagem celular de câncer de mama (MCF-7) foi demonstrado que o tratamento com DOX aumenta os níveis de ceramida em células sensíveis à DOX, mas os níveis não se alteram nas células resistentes à DOX, o que sugere que os níveis de ceramida podem estar envolvidos na resistência das células tumorais à DOX (DELPY et al., 1999; DONATO; KLOSTERGAARD, 2004; LUCCI et al., 1999; XU et al., 2016).

*1.1.3.2.4. Formação de radicais livres e indução de estresse oxidativo*

Atualmente é ponto esclarecido que sob condições adequadas a estrutura química das antraciclinas conduz à formação de radicais livres e indução de estresse oxidativo. A estrutura quinona da DOX pode sofrer uma redução eletrônica no anel C formando um radical semiquinona, reação mediada por flavoproteínas (Fp) adequadas que aceitam elétrons da nicotinamida adenina dinucleotídeo (NADH) ou da nicotinamida

adenina dinucleotídeo fosfato (NADPH) e os doam à DOX gerando o radical semiquinona (BERLIN; HASELTINE, 1981; MINOTTI et al., 2004; ŠIMŮNEK et al., 2009). Esses radicais são relativamente estáveis em ambientes anóxicos, entretanto em condições normóxicas, reagem rapidamente com o oxigênio, a quem doam seus elétrons, formando o ânion radical superóxido ($O_2^{\bullet-}$), que consequentemente pela ação da enzima superóxido dismutase (SOD), ou espontaneamente, produz peróxido de hidrogênio ($H_2O_2$). O $H_2O_2$, por sua vez, é uma molécula relativamente estável sob condições fisiológicas, sendo seu equilíbrio mantido pela ação das enzimas catalase (CAT) e glutationa peroxidase (GPx). Entretanto o $O_2^{\bullet-}$ e o $H_2O_2$ são capazes de gerar outro radical, altamente tóxico para a as células, o radical hidroxila ($HO^{\bullet}$). Esse radical tem um papel importante nas reações de Haber-Weiss (Fenton) (Eq. 1), as quais ocorrem em baixa velocidade, a menos que catalisadas por metais de transição, especialmente o Ferro (Fe) (HALLIWELL; GUTERIDGE, 2007). As reações de Haber-Weiss catalisadas por Fe são divididas em dois passos:

1) O íon férrico ($Fe^{3+}$) é reduzido ao íon ferroso ($Fe^{2+}$) pelo $O_2^{\bullet-}$(Eq. 2)
2) A reação de Fenton ocorre entre o $Fe^{2+}$ e o $H_2O_2$ (Eq. 3)

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO^- + HO^{\bullet} \text{ (Eq. 1)}$$

$$O_2^{\bullet-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \text{ (Eq. 2)}$$

$$H_2O_2 + Fe^{2+} \rightarrow HO^- + HO^{\bullet} + Fe^{3+} \text{ (Eq. 3)}$$

Tendo em vista o papel catalítico do Fe livre na produção do $HO^{\bullet}$, os organismos possuem mecanismos que regulam a homeostase do Fe, tais como proteínas específicas para sua aquisição, transporte e estocagem. O pool citosólico de Fe, que corresponde ao Fe que transita entre o transportador transferrina e o estocado pela proteína ferritina, é regulado por proteínas reguladoras de Fe (IRPs), as quais controlam a expressão dos receptores de transferrina e ferritina (MLADENKA et al., 2006). Entretanto a DOX, através da produção de $O_2^{\bullet-}$, age sobre a liberação do Fe da

proteína ferritina, contribuindo para o aumento na quantidade de Fe livre a ativo, que dá continuidade para as reações que geram $HO^{\bullet}$ (MYERS, 1998; THOMAS; AUST, 1986).

Um segundo mecanismo, pelo qual o Fe promove estresse oxidativo envolve a formação de complexos DOX-Fe, uma vez que devido a sua estrutura química, a DOX possui elevada afinidade por Fe, demonstrando um potencial quelante (MYERS, 1998). Na presença de um sistema redutor, tais como citocromo P450 redutase, NADH desidrogenase, thióis de cisteína ou glutationa, dentre outros, o complexo DOX- $Fe^{3+}$ é reduzido a DOX- $Fe^{2+}$. Esse por sua vez pode reagir com o $O_2$ para formar $O_2^{\bullet-}$, que é dismutado em $H_2O_2$, o qual pela reação de Haber-Weiss resulta em $HO^{\bullet}$. Alternativamente, o complexo DOX-$Fe^{2+}$ pode reagir diretamente com o $H_2O_2$ produzindo $HO^{\bullet}$ diretamente (Figura 3).

O $HO^{\bullet}$ formado possui uma meia-vida muito curta e é extremamente reativo. Diferentemente de outras espécies reativas de oxigênio (ERO), como o $O_2^{\bullet-}$ e o $H_2O_2$, não possui um sistema enzimático que o neutralize, podendo causar sérios danos às células e suas macromoléculas, tais como a peroxidação de lipídios de membranas e organelas, oxidação de proteínas e dano ao DNA (HALLIWELL; GUTERIDGE, 2007; MUINDI et al., 1984).

Portanto, não há uma única teoria a respeito do mecanismo tóxico da DOX sobre o tecido cardíaco. O dano ao miocárdio foi primariamente relacionado ao aumento na geração de ERO e radicais livres (ŠIMŮNEK et al., 2009), o qual tem sido indicado e amplamente estudado como o principal mecanismo de cardiotoxicidade.

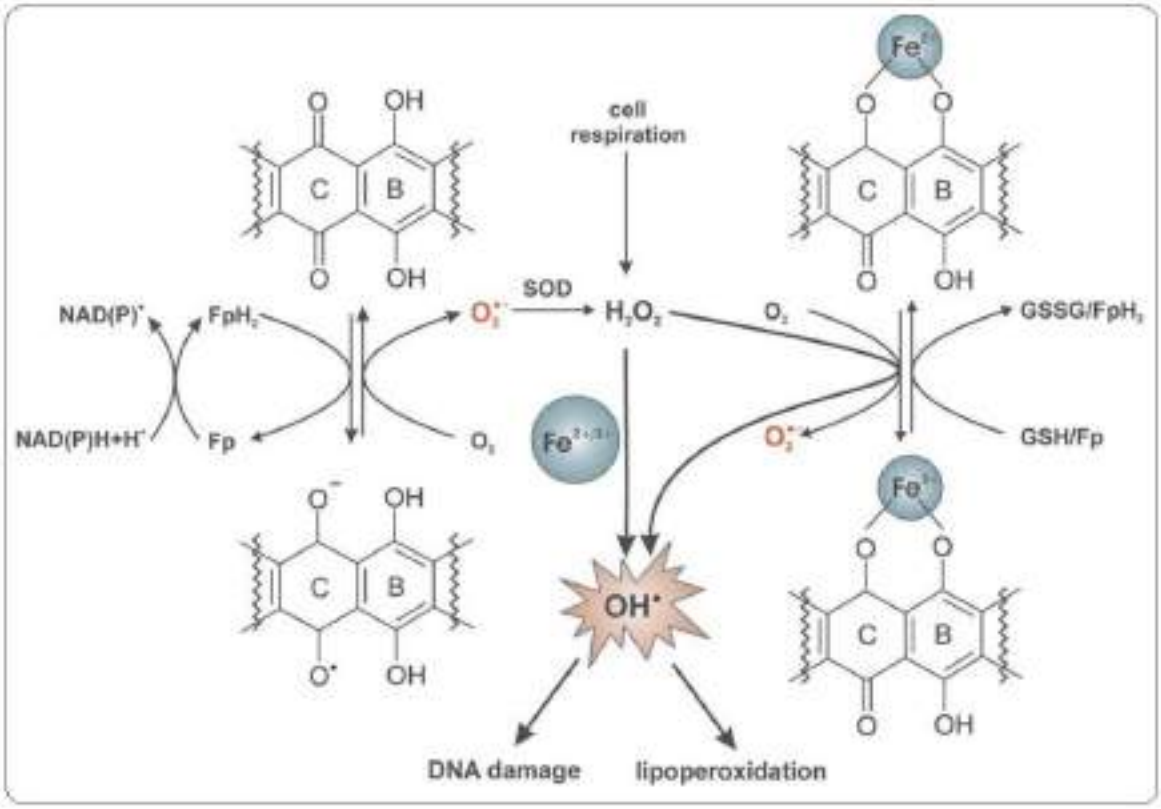


Figura 3: Mecanismo proposto para a formação de ERO pela DOX/antraciclinas que envolve íons Ferro ($Fe^{3+}/Fe^{2+}$), ânion radical superóxido ($O_2^{\bullet-}$), superóxido dismutase (SOD), peróxido de hidrogênio ($H_2O_2$), radical hidroxila ($HO^{\bullet}$), NAD(P) (nicotinamida adenina dinucleotídeo (fosfato)), Fp (flavoproteína), GSH/GSSG (glutationa reduzida/oxidada). (Reproduzido a partir de Tomáš Šimùnek et. al. Pharmacological Reports 61: 154–171, 2009).

### 1.1.3.3. Prevenção

A compreensão do mecanismo de cardiotoxicidade induzido pela DOX é de fundamental importância na busca de estratégias preventivas para combater o desenvolvimento de dano permanente ao tecido cardíaco. Tendo em vista que a cardiomiopatia induzida pela DOX tem como principal mecanismo a geração de radicais livres e ERO durante a metabolização mitocondrial da droga, grande parte das estratégias de prevenção tem como foco a redução do estresse oxidativo. A redução da cardiotoxicidade pode ser obtida através de inúmeros mecanismos, dentre eles encontra-se a administração de DOX na forma lipossomal peguilhada, que diminui a concentração de DOX livre na circulação, reduz sua velocidade de eliminação, aumenta a captação seletiva da substância pelas células tumorais e permite uma menor

frequência de administração do medicamento. O uso da DOX na forma lipossomal peguilhada demonstrou diminuir a cardiotoxicidade, mesmo em doses cumulativas > 500 mg/m$^2$ (O'BRIEN et al., 2004; RAFIYATH et al., 2012; SAFRA et al., 2000).

Estratégia adicional na redução do dano ao miocárdio pelo estresse oxidativo gerado pelas antraciclinas tem sido obtida com a utilização de agentes antioxidantes, tais como o probucol que tem demonstrado prevenir o dano pelo decréscimo na fração de ejeção do ventrículo esquerdo em modelos animais de cardiotoxicidade induzida pela DOX (LOU; DANELISEN; SINGAL, 2005; SINGAL et al., 2000; WALKER et al., 2011). O carverdilol, um beta-bloqueador adrenérgico, demonstrou ação protetora contra a disfunção ventricular esquerda causada pela DOX, o que também foi relacionado à sua ação antioxidante (OLIVEIRA et al., 2004; PEREIRA et al., 2011). Entretanto, embora muitos estudos realizados em modelos experimentais de cardiotoxicidade pela DOX em animais tenham comprovado os efeitos benéficos do tratamento com antioxidantes, nos estudos clínicos esses efeitos nem sempre são tão evidentes. Alguns agentes antioxidantes como a N-acetilcisteína, coenzima Q10, combinações de vitaminas E e C não foram avaliados em estudos clínicos comparativos e alguns pequenos estudos realizados não indicaram cardioproteção (VAN DALEN et al., 2008). Esse fato se deve aos mais variados agentes antioxidantes utilizados, ao tempo de tratamento, ao tipo de tumor e ao regime quimioterapia, dentre outros fatores (LADAS et al., 2004).

Como mencionado anteriormente, as ERO podem ser geradas pela interação entre a DOX e íons Fe na reação de Fenton. Tendo em vista esse mecanismo, o agente quelante de metais dexrazoxane foi desenvolvido com o intuito de prevenir e tratar a cardiotoxicidade induzida pela DOX. O dexrazoxane tem demonstrado em ensaios clínicos reduzir a incidência de ICC, mesmo em pacientes que já haviam recebido doses cumulativas de DOX > 300 mg/m$^2$ (SWAIN et al., 1997). Além disso, uma metanálise de nove estudos clínicos, incluindo um total de 1403 pacientes, descreve o papel protetor do dexrazoxane na insuficiência cardíaca (Risco Relativo (RR) 0,29, IC95% 0,20 a 0,41) (VAN DALEN et al., 2008). Entretanto, tendo em vista a concepção de que o dexrazoxane pode reduzir a eficácia terapêutica da DOX, é recomendado que

seu uso seja iniciado apenas após o paciente ter recebido uma dose cumulativa de DOX de 300 mg/m$^2$ (SWAIN et al., 1997; TRACHTENBERG et al., 2011).

## 1.2. COMPOSTOS BIOATIVOS DA DIETA E CARDIOPROTEÇÃO

### 1.2.1. Resveratrol

Compostos bioativos são constituintes da dieta normalmente encontrados em pequenas quantidades nos alimentos, que não são essenciais para a vida, mas que passaram a ser intensivamente estudados devido a seus efeitos benéficos sobre a saúde. Compostos como o resveratrol, flavonóides, fitoestrógenos, isotiocianatos, monoterpenos, licopeno, genisteína, curcumina e catequinas são exemplos de alguns dos compostos bioativos que podem ser obtidos a partir da dieta (KRIS-ETHERTON et al., 2002). Nesse sentido, são crescentes as evidências que comprovam que dietas ricas em vegetais, frutas, legumes e grãos, moderada ingestão de produtos lácteos, peixe e vinho, e pequena ingestão de carne vermelha e processada, o que caracteriza a dieta Mediterrânea, está associada à baixa mortalidade por doenças cardiovasculares (LOPEZ-GARCIA et al., 2014; PANAGIOTAKOS et al., 2015; TOGNON et al., 2014). Além disso, o consumo da dieta mediterrânea também está associado à baixa incidência de infarto agudo do miocárdio (IAM), doenças coronarianas, e acidente vascular cerebral (BUCKLAND et al., 2009; FUNG et al., 2009; GARDENER et al., 2011; TSIVGOULIS et al., 2015).

### 1.2.2. Efeito cardioprotetor do Resveratrol

O resveratrol é um fenol estilbenóide chamado trihidroxiestilbeno (Figura 4) encontrado especialmente na uva, frutas vermelhas e amendoim (SOLEAS; DIAMANDIS; GOLDBERG, 1997). É considerado uma fitoalexina, pois é produzido pelas plantas sob condições de estresse (LANGCAKE; PRYCE, 1976), como é o caso da síntese pelo fungo *Botrytis cinerea* presente na casca da uva, durante o processo de fermentação do vinho tinto. O resveratrol está presente em ambas as formas *cis/trans*,

entretanto é o isômero *trans* que possui maior efeito antioxidante e maior estabilidade biológica (BASLY et al., 2000; MIKULSKI; GÓRNIAK; MOLSKI, 2010). A presença do resveratrol, bem como outros compostos fenólicos, começou a atrair grande interesse a partir de 1992, quando foi lhe atribuído os efeitos cardioprotetores do vinho tinto (SIEMANN; CREASY, 1992). Desde então, muitos trabalhos tem demonstrado que o resveratrol pode prevenir ou retardar a progressão de inúmeras doenças, que incluem o câncer, doenças cardiovasculares, eventos isquêmicos, bem como o aumento da resistência ao estresse e tempo de vida desde leveduras até vertebrados (BERTELLI; DAS, 2009; HOWITZ et al., 2003; RAVAL et al., 2008; WAFFO-TÉGUO et al., 2001; YU; LI, 2012).

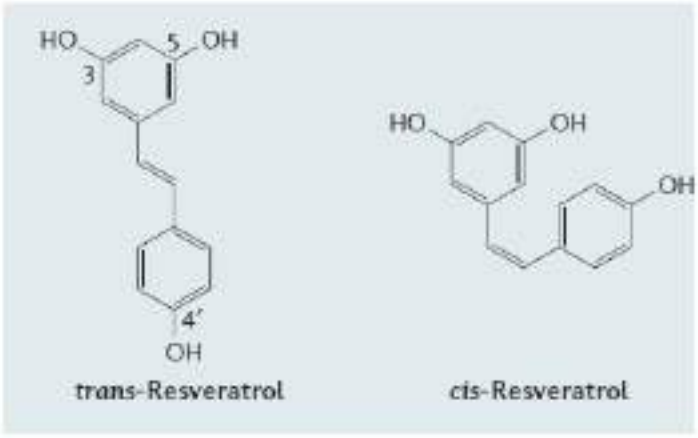


Figura 4: Estruturas do *trans*- e *cis*-Resveratrol. (Reproduzido a partir de Drug Discovery. Baur & Sinclair. Nature Reviews 5: 493–506, 2006).

### 1.2.3. Farmacodinâmica e efeitos fisiológicos do Resveratrol

O mecanismo pelo qual o resveratrol exerce seus efeitos benéficos nas mais variadas espécies e modelos de doenças ainda não está totalmente esclarecido. Resultados de estudos farmacocinéticos mostram que o resveratrol sofre ação da microbiota do intestino que produz os metabólitos dihidroxiresveratrol, 3,4'-dihidroxi-trans-estilbeno e 3,4'-dihidroxibibenzil (BODE et al., 2013). Em fase posterior de biotransformação, serão produzidos os metabólitos conjugados -3-orto-glicuronídeo, -4'-orto-glicuronídeo e -3-orto-sulfato (ALMEIDA et al., 2009; BOOCOCK et al., 2007), o

que ocorre rapidamente em menos de 2 horas após sua ingestão. As bactérias intestinais desempenham um papel importante no metabolismo do resveratrol o que contribui para a variação das frações de metabólitos entre os indivíduos (BODE et al., 2013; STEVENS; MAIER, 2016).

Fisiologicamente, o resveratrol apresenta um papel importante em muitas desordens e seus efeitos têm sido estudados em diferentes doenças. As pesquisas iniciaram com a descoberta de importantes efeitos cardiovasculares do resveratrol, mas desde então vários outros efeitos benéficos sobre a saúde foram identificados, tais como: no tratamento da fibrilação atrial (BACZKÓ; LIGHT, 2015); como anti-diabetogênico (BAGUL; BANERJEE, 2015); aumentando a expectativa de vida, e protegendo contra o envelhecimento e doenças relacionadas (RAMIS et al., 2015; VAHID et al., 2015); na regulação do câncer e doenças neurodegenerativas (AIRES; DELMAS, 2015), dentre outros.

Devido ao papel do estresse oxidativo nas doenças cardiovasculares, maior atenção tem sido dada ao uso de antioxidantes naturais no seu tratamento. Nessa linha, o resveratrol tem demonstrado efeito antiapoptótico, responsável em parte, por seu papel cardioprotetor, uma vez que ao aumentar a resistência contra o estresse oxidativo por neutralizar o $H_2O_2$, o resveratrol previne a morte de células endoteliais (UNGVARI et al., 2007). Outros efeitos tais como, ação anti-inflamatória (JØRAHOLMEN et al., 2015; TROTTA et al., 2016), antiproliferativa (AMIRI et al., 2013; FERRUELO et al., 2014; KUO; CHIANG; LIN, 2002), antiangiogênica (EL-AZAB et al., 2011; KASIOTIS et al., 2013), também tem sido atribuídas aos mecanismos protetores do resveratrol, entretanto a modulação do estresse oxidativo tem sido destacada como um dos seus principais efeitos, por estar correlacionada com os demais. Dessa forma, o resveratrol pode apresentar efeitos benéficos sobre inúmeras doenças, particularmente àquelas em que o estresse oxidativo apresente um papel importante.

## 1.3. EXERCÍCIO

### 1.3.1. Exercício como agente cardioprotetor

A busca por estratégias adicionais para controle e prevenção dos efeitos cardiotóxicos dos quimioterápicos tem crescido consideravelmente nas últimas décadas. Particularmente, estratégias farmacológicas baseadas em agentes naturais, com menores efeitos colaterais, com menor custo e mais acessíveis, como o encontrado em componentes bioativos naturais, que podem ser obtidos através de dieta tem recebido atenção especial (AGGARWAL et al., 2011).

Outras estratégias, não farmacológicas, baseadas na mudança do estilo de vida, também têm sido observadas como importantes moduladoras da função cardíaca e proteção contra o dano ao miocárdio em diferentes situações. Nesse sentido, a cardioproteção miocárdica pelo exercício físico contra diferentes insultos cardíacos é atualmente um efeito reconhecido. Quando o exercício é realizado de forma moderada e sistemática, pode constituir uma excelente ferramenta tanto para o tratamento quanto para a prevenção de diversas doenças, onde estão incluídas as doenças cardiovasculares (DUNCKER; BACHE; MERKUS, 2012; DUNCKER; BACHE, 2008).

O exercício, quando praticado na forma de condicionamento físico é capaz de gerar um processo de adaptação positiva ao coração. Dentre as respostas adaptativas ao exercício, são observadas bradicardia ao repouso e ao exercício em intensidade submáxima; aumento na dimensão diastólica final; melhora da função ventricular e aumento na resistência do coração aos eventos isquêmicos e estímulos deletérios que causem estresse oxidativo e apoptose (ASCENSÃO; FERREIRA; MAGALHÃES, 2007; MOORE, 1998; POWERS; QUINDRY; KAVAZIS, 2008). Além disso, o condicionamento com exercício físico torna o miocárdio menos suscetível aos efeitos deletérios de episódios isquêmicos agudos; além de ter demonstrado ser efetivo no tratamento e/ou prevenção dos déficits cardíacos funcionais decorrentes da hipertensão crônica, idade avançada e de IAM (HEINONEN et al., 2014; MEZZANI; CORRÀ; GIANNUZZI, 2008; PARKER; KALASKY; PROCTOR, 2010; TOTH et al., 2012; VELLA; ROBERGS, 2008).

### 1.3.2. Efeitos fisiológicos do exercício sobre as ERO

Embora o exato mecanismo de cardioproteção pelo exercício continue em discussão, tem sido normalmente associado ao decréscimo na produção de ERO e ao aumento na resposta de diversos sistemas de defesa antioxidante (ASCENSÃO et al., 2003; JI, 2002; LEE et al., 2012b; POWERS et al., 2014). Nesse sentido, foi demonstrado que o exercício induz ao aumento da atividade das enzimas antioxidantes cardíacas e do conteúdo de glutationa, melhora a função mitocondrial e reduz a formação de peróxidos lipídicos (ASCENSÃO et al., 2005b; VENDITTI; DI MEO, 1996), além de aumentar a expressão de proteínas cheperonas (ASCENSÃO et al., 2005b; POWERS et al., 1998).

Relativamente à cardiotoxicidade induzida pela DOX, desde as investigações iniciais, na qual ratos tratados com DOX submetidos a uma sessão aguda de nado forçado apresentaram menores taxas de toxicidade e mortalidade comparativamente aos controles (COMBS; HUDMAN; BONNER, 1979), diversos trabalhos foram realizados para analisar também os efeitos do exercício crônico sobre o dano cardíaco causado pela DOX. Desde então, inúmeros estudos comprovaram os efeitos benéficos do exercício sobre o sistema cardiovascular, sendo capaz de atenuar os efeitos cardiotóxicos do tratamento agudo ou crônico com DOX e protegendo o tecido cardíaco contra o estresse oxidativo, dano e morte celular (ASCENSÃO; OLIVEIRA; MAGALHÃES, 2012; ASCENSÃO et al., 2005a, 2005b, 2006; MARQUES-ALEIXO et al., 2015).

## 1.4. EPIGENÉTICA

### 1.4.1. Memória epigenética em mamíferos

O termo *epigenética* deriva do prefixo grego *epi*, que significa *acima*, definindo o fenótipo herdável resultante de modificações que ocorrem na cromatina (“acima” ou “para além” dos genes) sem alterações na sequência primária do DNA (BERGER et al., 2009). O termo foi originalmente empregado por Conrad Hal Waddington que a definiu

como “interações dos genes com o seu ambiente e que dão forma ao fenótipo” (WADDINGTON, 1940). Posteriormente, a epigenética passou a ser compreendida como “as alterações herdáveis na expressão dos genes que dão forma ao fenótipo e que ocorrem independentemente de alterações na sequência do DNA” (BONASIO; TU; REINBERG, 2010; DAXINGER; WHITELAW, 2012).

Uma memória epigenética pode ser criada e a informação transmitida de uma geração à outra através de padrões de herança que se baseiam em modificações na estrutura do DNA, tais como metilação de resíduos de citosina, modificações de histonas e de miRNAs (BONASIO; TU; REINBERG, 2010; WHITELAW; WHITELAW, 2008), como demonstrado na Figura 5. Essas informações podem ser propagadas entre as gerações durante a gametogênese e embriogênese precoce, gerando os efeitos epigenéticos fenotípicos inter/transgeracionais, os quais não podem ser explicados pela genética Mendeliana (ou por alterações na sequência primária do DNA). Isso inclui, por exemplo, os efeitos de exposições ambientais sobre adultos que alteram o fenótipo do embrião em desenvolvimento via placenta; ou o recém-nascido via leite materno (DAXINGER; WHITELAW, 2012; WHITELAW; WHITELAW, 2008). A habilidade dos mamíferos de transmitir uma informação epigenética à sua prole evidencia claramente que a hereditariedade não está restrita à sequência do DNA e que a epigenética possui um papel importante na produção de descendentes saudáveis.

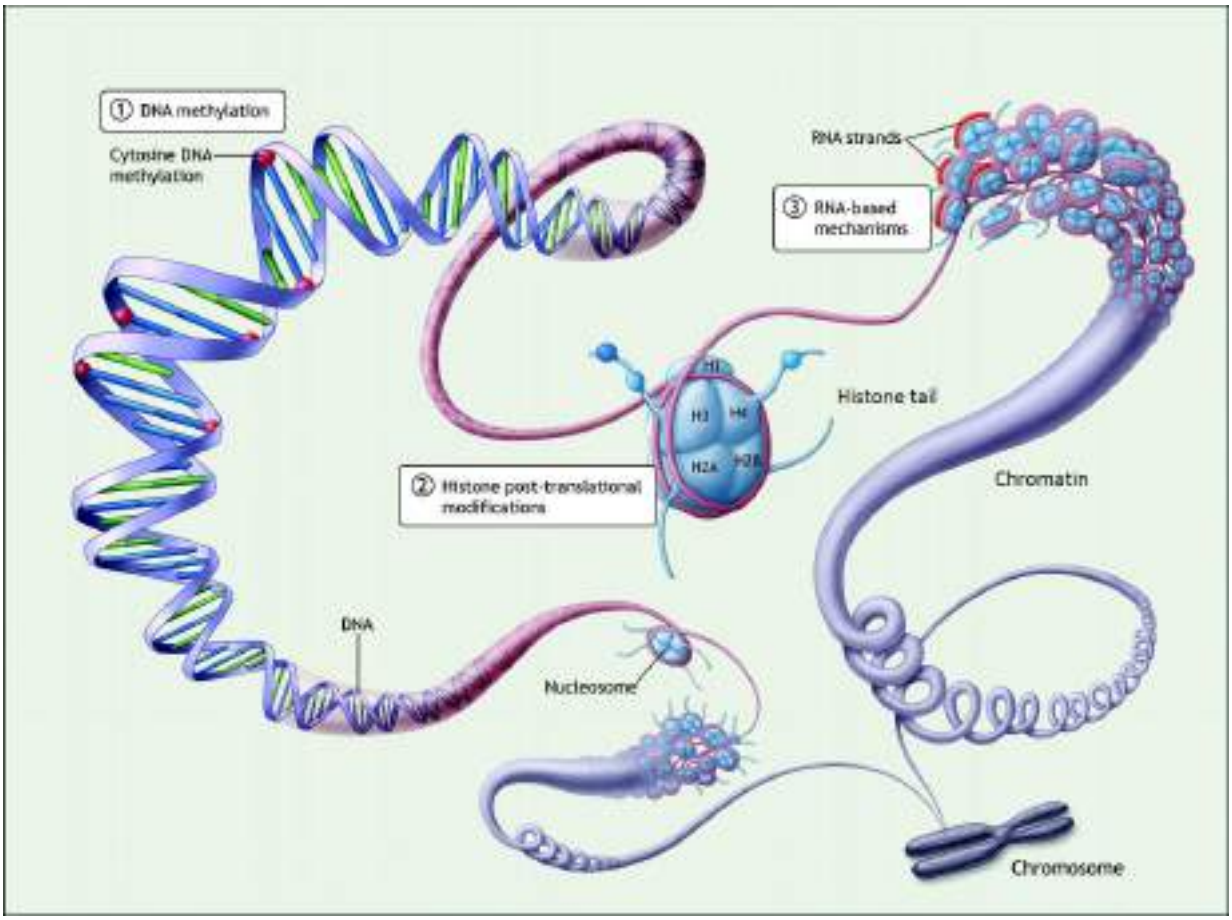


Figura 5: Mecanismos epigenéticos de regulação gênica. (Reproduzido a partir de Matouk & Marsden. Circ. Res. 102: 873-887, 2008).

### 1.4.2. Modificações epigenéticas e as Desacetilases de Histonas

Nas células eucarióticas o DNA genômico está mantido no complexo DNA-protéico da cromatina, onde as proteínas predominantes são as histonas. O principal elemento de repetição da cromatina são os nucleossomos, que consistem de 147 pares de bases do DNA envoltos por um octâmero de proteínas histonas (H2A, H2B, H3 e H4) (WOLFFE, 1992). Essas proteínas fornecem uma estrutura ao genoma e também permitem que a cromatina seja modificada em determinadas situações. Esse é o caso das modificações de histonas, que alteram a ligação dos fatores de transcrição aos promotores do gene alvo, modificando a expressão gênica (BROWNELL; ALLIS, 1996; WOLFFE, 1999).

Dentre as principais modificações epigenéticas destacam-se:

1) as modificações químicas ao nível dos nucleotídeos, que incluem a metilação do DNA e o RNA interferência (RNAi);

2) as modificações ao nível das histonas, como as modificações pos-traducionais de proteínas histonas e a incorporação de variantes de histonas; e

3) remodelamento de nucleossomos, processo dependente de ATP, que regula a acessibilidade ao DNA nucleossomal (GRAFF et al., 2011; MATOUK; MARSDEN, 2008).

Relativamente ao processo de modificação de proteínas histonas, as modificações pos-traducionais podem ocorrer em todas as histonas, principalmente na cauda $NH_2$-terminal, consistindo dos processos de acetilação, metilação, fosforilação, ubiquitinação e sumoilação (GRAFF et al., 2011). Devido as suas propriedades químicas, essas modificações epigenéticas podem alterar a compactação da cromatina, e como consequência, a acessibilidade da maquinaria de transcrição ao DNA.

Dentre as modificações de proteínas histonas, o processo de acetilação tem recebido destaque. As histonas podem ser acetiladas em seus resíduos de lisina (K), onde o processo de acetilação tem sido relacionado ao processo de ativação do gene e início da transcrição, uma vez que a acetilação deixa a estrutura da cromatina menos compactada e permite o recrutamento e ligação de fatores de transcrição e da RNA polimerase (BROWNELL; ALLIS, 1996). As enzimas que regulam o processo de acetilação de histonas são as Acetil-transferases de Histonas (HATs) e as Desacetilases de Histonas (HDACs), as quais regulam o estado da cromatina de forma a estar hora descompactada, acetilada e acessível, ou mais compactada, desacetilada e inacessível aos fatores de transcrição (Figura 6).

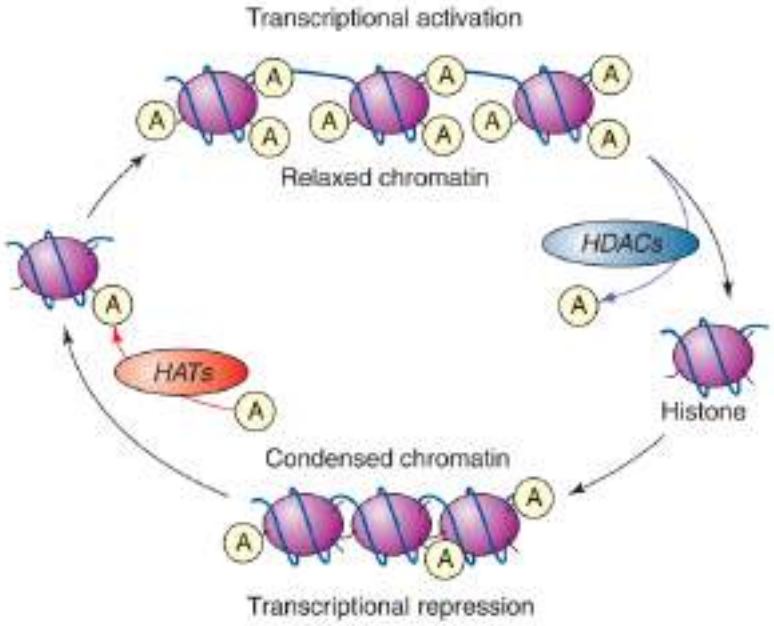


Figura 6: Regulação do estado transcricional da cromatina pelas Acetil-transferases de Histonas (HATs) e Desacetilases de Histonas (HDACs). (Reproduzido a partir de Chuang et al. Trends Neurosci. 32: 591–601, 2009).

As HDACs são enzimas conservadas através da evolução de muitas espécies. Em humanos, as HDACs podem ser divididas em 4 classes, o que teve como base sua homologia com as HDACs de leveduras (FRYE, 2000). As HDACs classe I incluem as HDAC1, 2, 3 e 8 relacionadas com a enzima desacetilase de levedura Rpd3. As HDACs classe II incluem as HDAC4, 5, 6, 7, 9 e 10 relacionadas a proteína de levedura HDA1. As HDACs classe I e II têm sido mais extensivamente estudadas por seus papéis no Sistema Nervoso Central (SNC) (LU et al., 2000; MORRIS; MONTEGGIA, 2013). As HDACs classe III são desacetilases com função dependente de $NAD^+$ e são conhecidas como “Sirtuínas” por sua homologia com a HDAC Sir2 de levedura. Ainda, a HDAC classe IV compreende a HDAC11, a isoforma identificada mais recentemente e ainda pouco estudada (VOELTER-MAHLKNECHT; HO; MAHLKNECHT, 2005).

As sirtuínas compreendem uma família de enzimas, Sirt1 – Sirt7, que possuem dentre outras características, localizações subcelulares, atividade e alvos distintos na célula (FRYE, 2000; MICHISHITA et al., 2005; MORRIS, 2013; SEBASTIÁN; MOSTOSLAVSKY, 2015), como ilustrado na Tabela 2 abaixo.

Tabela 2: Atividade e localização das sirtuínas.

| Sirtuin | Activity | Location | Targets |
|---|---|---|---|
| SIRT1 | Deacetylation | Nucleus<br>Cytosol | FOXO1, FOXO3, PGC-1α, p53, NF-κB, Notch, HIF1α, LXR, FXR, SREBP1c, etc |
| SIRT2 | Deacetylation | Cytosol | FOXO1, PEPCK, tubulin, PAR-3 |
| SIRT3 | Deacetylation | Mitochondria<br>Nucleus | OXPHOS complexes, SOD2, LCAD, HMGCS2, GDH, IDH2, PIP2, ACADL, FOXO3, ACSS2, OTC, GLUD1, NDUFA9, SDHA, ATP5A1, ALDH2, MRPL10, STK11, HISTH3, XRCC6 |
| SIRT4 | ADP-ribosylation<br>Deacetylation | Mitochondria<br>Cytosol | GDH |
| SIRT5 | Deacetylation<br>Demalonyation<br>Desuccinylation | Mitochondria | CPS1 |
| SIRT6 | Deacetylation<br>ADP-ribosylation | Nucleus | H3K9, H3K56 |
| SIRT7 | Deacetylation | Nucleus<br>Cytosol | H3K18ac<br>HIF-1α/HIF-2α |

(Reproduzido e adaptado a partir de Brian J. Morris. Free Rad Biol Med 56: 133–171, 2013; & Carlos Sebastián & Raul Mostoslavsky. Seminars in Cell & Developmental Biology 43: 33–42, 2015).

Apesar das sirtuínas possuírem a função enzimática de desacetilase de histonas, algumas possuem atividades adicionais; tais como a Sirt6 e a Sirt4 que agem ainda como mono-ADP-ribosiltransferases, numa reação onde a molécula de ADP-ribosil é transferida do $NAD^+$ para um substrato protéico (BORDO, 2013). Particularmente a Sirt6, desempenha um papel chave no reparo do DNA e na manutenção da estabilidade do genoma, especialmente integrando ações de fatores de sinalização de dano ao DNA com o recrutamento e ativação de enzimas de reparo, sobretudo em situações de estresse oxidativo (MAO et al., 2011).

1.4.3. A influência do ambiente na formação do epigenoma - Efeitos epigenéticos do exercício e da nutrição

Até poucos anos atrás o conhecimento estabelecido era de que a informação hereditária era transmitida entre as gerações apenas pela sequência do DNA, e que qualquer alteração nessa sequência era aleatória e independente de fatores

ambientais. Entretanto, durante as duas últimas décadas o aumento nas pesquisas no campo da epigenética desmistificou esse conhecimento ao demonstrar que a herança da sequência do DNA não é a única forma de transmissão de informações de traços físicos, comportamentais e emocionais entre mamíferos. Além disso, foi recentemente estabelecido que o epigenoma pode ser modulado por uma variedade de fatores ambientais, tais como agentes químicos; nutricionais; o ambiente dos anos iniciais de vida; cuidado materno; bem como pela prática de atividade física (ABEL; RISSMAN, 2013; ANWAY; LEATHERS; SKINNER, 2006; BLAZE; SCHEUING; ROTH, 2013; BURDGE et al., 2007; NTANASIS-STATHOPOULOS et al., 2013; ROTH; SWEATT, 2011; WATERLAND et al., 2006; WEAVER et al., 2004). Dessa forma o epigenoma se constitui pela interface entre e os genes e o ambiente, podendo ser visto como um potencial mecanismo para as adaptações inter- e transgeracionais originadas pelo ambiente.

Relativamente ao papel do exercício, é crescente o número de trabalhos demonstrando que esse é capaz de modular parâmetros epigenéticos; como por exemplo, a alteração do perfil de acetilação de histonas e consequentemente aumento do processo de transcrição de genes relacionados a funções específicas do SNC (ELSNER et al., 2011; GOMEZ-PINILLA et al., 2011). Nesse sentido, Elsner e cols. demonstraram recentemente que diferentes protocolos experimentais de exercício em esteira podem modular as atividades das enzimas HATs e HDACs no hipocampo de ratos (ELSNER et al., 2011). Além desses, outros estudos tem demonstrado a relação existente entre modificações epigenéticas induzidas pelo exercício e: melhor prognóstico do diabetes tipo 2 (DOS SANTOS et al., 2015); maior plasticidade sináptica devido a regulação da expressão do BDNF hipocampal (GOMEZ-PINILLA et al., 2011); redução de déficits cognitivos (ELSNER et al., 2013) e do declínio de memória relacionados à idade, pela redução do perfil neuroinflamatório (LOVATEL et al., 2012, 2013a); dentre outros efeitos.

A nutrigenômica é outra área que tem recebido destaque, ao explorar e definir o envolvimento da dieta na formação do epigenoma. Sendo a nutrição a maior exposição ambiental que influencia significativamente na vida e expectativa de vida (RAKYAN et al., 2011), os nutrientes recebem um papel de destaque pela capacidade de modular

mecanismos epigenéticos (PHAM; LEE, 2012). O interesse nos efeitos epigenéticos transgeracionais dos componentes da dieta foi motivado pelas observações de que camundongos *Agouti* alimentados com dieta rica em polifenóis da soja apresentaram alterações nos padrões de metilação do DNA em suas ninhadas e os protegeram contra o diabetes, obesidade e câncer, o que foi observado através de múltiplas gerações (DOLINOY; HUANG; JIRTLE, 2007; DOLINOY et al., 2006). Assim, inúmeros compostos bioativos derivados da dieta, tais como: o resveratrol, a curcumina, os polifenóis do chá verde, dentre outros, podem direta ou indiretamente modular parâmetros epigenéticos interagindo com enzimas que catalisam a metilação do DNA ou as modificações de histonas (CHOI; FRISO, 2010). Essas modificações podem acumular-se no tempo e estar envolvidas na patogênese ou na proteção de inúmeras doenças, como as relacionadas ao envelhecimento, o diabetes, o câncer e as doenças cardiovasculares (AAGAARD-TILLERY et al., 2008; DE FOURMESTRAUX et al., 2004).

## 2. JUSTIFICATIVA

A DOX está entre os quimioterápicos da classe das antraciclinas de uso mais frequente em oncologia. Entretanto seu uso não está livre de complicações, onde o desenvolvimento de resistência em células tumorais e a toxicidade não específica em tecidos saudáveis apresenta uma incidência elevada, destacando-se a toxicidade cardíaca, desenvolvimento de cardiomiopatia e ICC como efeitos colaterais mais comuns.

A cardiotoxicidade crônica tardia é particularmente relevante nos casos de sobreviventes adultos de tumores pediátricos, onde até 65% dos sobreviventes de tumores malignos na infância tratados com DOX/antraciclinas podem apresentar evidências ecocardiográficas de alterações na função contrátil do ventrículo esquerdo. A ocorrência de disfunção cardíaca após tratamento com DOX em pacientes de qualquer idade pode chegar a 30%; complicação que pode determinar interrupção do tratamento quimioterapêutico e comprometer a cura ou o adequado controle do câncer.

Embora os mecanismos de citotoxicidade da DOX sejam diversos, o dano ao miocárdio foi primariamente relacionado ao aumento na geração de ERO e radicais livres. Esse fato que fez com que grande parte das estratégias de prevenção tivesse como foco a redução do estresse oxidativo, destacando-se a utilização de agentes antioxidantes.

O resveratrol é um composto bioativo que pode ser obtido em pequenas quantidades na dieta, apresentando dentre outras propriedades, a antioxidante, anti-inflamatória, antiproliferativa, antiangiogênica e antiapoptótica e que começou a atrair grande interesse a partir de 1992, quando foi lhe atribuído os efeitos cardioprotetores do vinho tinto. Outras estratégias, não farmacológicas, baseadas na mudança do estilo de vida, também têm sido observadas como importantes moduladoras da função cardíaca. Esse é o caso da cardioproteção pelo exercício físico, o qual tem sido associado ao decréscimo na produção de ERO e ao aumento na resposta de diversos sistemas de defesa antioxidante em diferentes situações de dano ao miocárdio.

Além dos efeitos benéficos do resveratrol e do exercício como antioxidantes e cardioprotetores, pesquisas recentes tem demonstrado que tanto o resveratrol – um composto bioativo da dieta, quanto o exercício – um componente do estilo de vida, são fatores ambientais com potencial para modulação de fatores epigenéticos. Modulação essa, que pode criar uma memória epigenética e ser transmitida de uma geração à outra, gerando os efeitos epigenéticos fenotípicos inter- e transgeracionais.

Dessa forma, tendo em vista os efeitos cardioprotetores do exercício e do resveratrol e as evidências crescentes de seus envolvimentos na modulação de fatores epigenéticos, esse trabalho teve como objetivo investigar os efeitos intergeracionais do exercício e da suplementação com resveratrol sobre a toxicidade da DOX no coração da prole, examinando a possiblidade de herança de efeitos cardioprotetores pelo exercício e resveratrol.

# 3. OBJETIVOS DO ESTUDO

## 3.1. Objetivo Geral

Verificar os efeitos intergeracionais do exercício físico e da suplementação com resveratrol sobre a toxicidade da doxorubicina nos cardiomiócitos da ninhada.

## 3.2. Objetivos Específicos

Avaliar os efeitos do exercício físico em esteira e da suplementação com resveratrol realizados durante o período gestacional da genitora sobre o coração da ninhada. Para esse objetivo, a cultura primária de cardiomiócitos de cada ninhada de ratos neonatos foi tratada com diferentes concentrações e tempos de exposição à DOX, e os seguintes objetivos foram visualizados:

- ✓ Avaliar a viabilidade dos cardiomiócitos expostos DOX;
- ✓ Investigar o mecanismo de morte celular induzida pela DOX nos cardiomiócitos da ninhada;
- ✓ Avaliar o estresse oxidativo gerado nos cardiomiócitos pela exposição à DOX;
- ✓ Investigar o dano ao DNA gerado pela DOX nos cardiomiócitos da ninhada e a presença de dano oxidativo nas bases do DNA;
- ✓ Analisar a capacidade de defesa antioxidante enzimática e não enzimática dos cardiomiócitos expostos à DOX;
- ✓ Avaliar o nível de expressão da desacetilase de histona Sirt6 nos cardiomiócitos expostos à DOX;
- ✓ Conferir se há efeitos intergeracionais do exercício e do resveratrol, com uma cardioproteção sendo transferida de genitora para prole.

4. **CAPÍTULO 1: ARTIGO 1:** Exercise during pregnancy decreases doxorubicin-induced cardiotoxic effects on neonatal hearts.

**Exercise during pregnancy decreases doxorubicin-induced cardiotoxic effects on neonatal hearts.**

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# Exercise during pregnancy decreases doxorubicin-induced cardiotoxic effects on neonatal hearts

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ABSTRACT

Cancer treatment with Doxorubicin (DOX) is limited due its dose-dependent cardiotoxicity, mainly related to the oxidative stress production. In experimental models of DOX treatment exercise can be used as a beneficial adjuvant therapy. This work aimed to investigate the effects of exercise during pregnancy on DOX-induced cardiotoxicity in cardiomyocytes of progeny, examining the possible intergenerational cardioprotective effects of maternal exercise. For this purpose pregnant rats were divided in control and exercise groups and pre-treated during gestational days. Hearts of newborns were used to obtain a culture of cardiomyocytes to be treated with DOX for analyses of cell viability, apoptosis and necrosis; ROS production; DNA damage; SOD and CAT activities; and Sirt6 protein expression. The results showed that exercise during pregnancy induced an increase in the viability of neonatal cardiomyocytes and a decrease in DOX-induced apoptotic and necrotic death which were correlated to the decrease in ROS production and an increase in antioxidant defenses. Exercise also protected neonatal cardiomyocytes from DOX-induced DNA damage, demonstrating a reduction in the oxidative DNA breaks. Likewise, exercise induced an increase in expression of Sirt6 in neonatal cardiomyocytes. Therefore, these results demonstrate for the first time that exercise performed by mothers protects the neonatal heart against DOX-induced toxicity. Our data demonstrate the intergenerational effect of exercise in cardiomyocytes of progeny, where the modulation of oxidative stress through antioxidant enzymes, and DNA integrity via Sirt6, were induced due to exercise in mothers, increasing the resistance of the neonatal heart against DOX toxicity.



## 1. Introduction

The chemotherapeutic drug doxorubicin (DOX) is a highly effective anthracycline antitumor antibiotic, commonly used to treat many types of cancer (Datta et al., 2015). In affected cells, the cytotoxic action of DOX involves DNA intercalation and the formation of additional bonds between nitrogen bases of DNA strands, compromising the replication and transcription process. However, the use of DOX in the treatment of human tumors is limited due to its strong cardiotoxicity leading to a dose-dependent cardiomyopathy and heart failure (Smith et al., 2016; Volkova and Russell, 2011).

Multiple mechanisms are involved in the DOX-associated cardiotoxicity. The most commonly accepted mechanism is the iron-mediated formation of reactive oxygen species (ROS), which promotes myocardial oxidative stress (Cascales et al., 2012; Rochette et al., 2015). This ROS generation can cause damage to lipid membranes and nucleic acids, which together with reduced levels of antioxidants and sulfhydryl groups leads to the initiation of apoptosis in cells (Alexieva et al., 2014; Chao et al., 2011; Octavia et al., 2012). In addition, the mitochondria plays an essential role in the pathogenesis of DOX-induced cardiotoxicity due its function in the maintenance of myocardial tissue homeostasis. It has been demonstrated that DOX causes an impairment of mitochondrial

Abbreviations: DOX, doxorubicin; Sirt6, sirtuin6; HDAC, histone deacetylase; FPG, formamidopyr midine DNA glycosylase; Endo III, endonuclease III; PARP, poly-ADP-ribose polymerase; NAD+, nicotinamide adenine dinucleotide; SOD, superoxide dismutase; ROS, reactive oxygen species; CAT, catalase; Annexin V-PE, annexin V-phycoerythrin; 7-AAD, 7-amino-actinomycin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; LMP, low melting-point; HMP, high melting-point; H2DCF-DA, 2′-7′-dihydrodichlorofluorescein diacetate; DCF, 2′-7′-dichlorofluorescein; TB, trypan blue; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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respiration (Carvalho et al., 2010; Lipshultz et al., 2016) and inhibition of the oxidative phosphorylation, which involves a metabolic remodeling between the aerobic fatty acid oxidation and the anaerobic glycolysis (Carvalho et al., 2010). Conversely, the stimulation of basal mitochondrial respiration is able to decrease DOX-induced apoptotic signaling in cardiomyoblasts (Deus et al., 2015). This indicates that the protection of mitochondrial function can be beneficial for counteracting the DOX-induced cardiotoxic effects (Pereira et al., 2011) that ultimately leads to cardiomyocyte cell death and consequent cardiovascular dysfunction.

Notwithstanding this, the prevention of DOX-induced cardiomyopathy is still unsolved and the only truly effective method to prevent its cardiotoxicity is a dose-limiting approach that may compromise its chemotherapeutic properties (Gratia et al., 2012). To reduce the cardiac side effects of DOX-treatment, researchers have investigated adjuvant therapies using animal models. Among a number of non-pharmacological strategies, physical exercise of different types and features has been studied. Both acute and chronic models of exercise trigger a preconditioning effect on DOX-treated rats that protects cardiac tissue and especially mitochondria against the drug-induced negative remodeling (Ascensão et al., 2012, 2011a, 2011b). Moreover, studies suggest that exercise acts against the damaging consequences of *in vivo* and *in vitro* DOX treatment on rodent hearts either by preventing or attenuating the toxicity, and these effects are mainly related to enhancement in the antioxidant defenses and the decrease of the ROS production and apoptosis (Ascensão et al., 2005a, 2005b; Marques-Aleixo et al., 2015). Regardless of the valuable action of exercise, the improvement of aerobic capacity in patients undergoing adjuvant therapy is small, due in part to low adherence to programs of exercise (Jones et al., 2011).

Exercise has preventive/therapeutic benefits on many pathological and physiological situations. During pregnancy, in the absence of obstetric complications, moderate exercise could be beneficial to the maternal–fetal unit and infant through improved physiological, metabolic and psychological parameters, along with reduced risk of morbidity and mortality (Marques et al., 2014; Melzer et al., 2010; Prather et al., 2012). Experimental models have demonstrated the beneficial effects of maternal exercise during pregnancy to offspring, such as the improvement of mitochondrial function and biogenesis in the brain of the offspring (Park et al., 2013), an increase in object recognition memory (Robinson and Bucci, 2014), and a decrease in the risk of mammary tumorigenesis (Camarillo et al., 2014). Moreover, it has also been shown that exercise prevents maternal high-fat diet-induced hypermethylation of the Pgc-1α gene and age-dependent metabolic dysfunction in the offspring (Laker et al., 2014), demonstrating the protective intergenerational effects of exercise by mothers.

In recent decades interest in a family of proteins named as sirtuins has increased because of their role as regulators involved in numerous cellular signaling pathways. Sirtuins are a family of seven members (Sirt1 to Sirt7) of nicotinamide adenine dinucleotide ($NAD^+$)-dependent histone deacetylases implicated in the regulation of multiple pathophysiological processes, including oxidative stress, DNA damage repair, cell metabolism, apoptosis, tumorigenesis, neurodegeneration, and aging (Barreiro and Gea, 2015; Lawless et al., 2010; Morris, 2013). Of particular interest is the histone deacetylase Sirt6 as a scaffold protein in DNA damage repair. This histone deacetylase is a chromatin-bound protein that is rapidly recruited to sites of double strand breaks following DNA damage, acting on critical steps required for proper recruitment of downstream DNA damage repair factors and efficient repair (Cai et al., 2012; Toiber et al., 2013).

Deacetylation is the main function of sirtuins, however some of them have deacylase, adenosine diphosphate-ribosylase, demalonylase, glutarylase, and desuccinylase properties (Morris, 2013). Sirtuins can be activated upon exercise or caloric restriction, and control critical cellular processes in the nucleus, mitochondria and cytoplasm, such as the maintenance of metabolic homeostasis, reduction of cellular damage and inflammation, all of which protect against a range of age-related diseases, including cardiovascular pathologies. In cardiovascular diseases, sirtuins have gained interest by their protective effects. Particularly, Sirt6 is seen to play an important role in cardiovascular disease including cardiac hypertrophy, heart failure and myocardial hypoxic damage (Cai et al., 2012; Maksin-Matveev et al., 2015; Sundaresan et al., 2012). Moreover, previous experimental research demonstrated that exercise is able to change Sirt6 and Sirt1 levels in skeletal muscle of aged rats (Huang et al., 2016; Koltai et al., 2010a).

In view of the evidence about the protective effects of exercise on DOX-induced cardiotoxicity and the beneficial effects to offspring of exercise by mothers, this study aimed to investigate the effects of exercise during pregnancy on DOX-induced cardiotoxicity in the hearts of progeny, examining the possible intergenerational cardioprotective effects of maternal exercise.

## 2. Methods

### 2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (SFB) and penicillin/streptomycin were obtained from Gibco-BRL (Grand Island, NY, USA). Low melting-point agarose (LMP), normal melting-point agarose (NMP), 2′-7′-dihydrodichlorofluorescein diacetate ($H_2$DCF-DA), 2′-7′-dichlorofluorescein (DCF), trypan blue (TB), gelatin, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS) and pancreatin were purchased from Sigma (St. Louis, MO, USA). Primary antibodies anti-sirt6 (ab62739) and anti-actin (C-2) (sc-8432) were purchased from Abcam (UK) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Annexin V-Phycoerythrin (PE) and 7-Amino-Actinomycin (7-AAD) were purchased from BD Biosciences (San Diego, CA). Formamidopyrimidine DNA-glycosylase (FPG) and endonuclease III (EndoIII) were obtained from BioLabs (New England, USA). All other reagents were of analytical grade and purchased from local commercial suppliers.

### 2.2. Ethical approval and animals

The research followed the ethical rules established by the Brazilian Guidelines for the Care and Use of Animals for Scientific and Didactic Purposes (DOU 27/5/13, MCTI, p.7). All procedures outlined in this study were approved by the Research Ethics Committee of the Federal University of Health Sciences of Porto Alegre (CEUA number 182/13).

Female (n = 16) and male (n = 8) albino Wistar rats (aged 4 weeks and weighing 70–100 g) from the Center for the Reproduction of Laboratory Animals of the UFCSPA, were kept under a day/night cycle (lights on 7:00 am to 7:00 pm), room temperature 23 °C ± 1, and 50% ± 5 relative humidity. Throughout the experiment the animals received a standard pellet diet (Nuvital CR1®, Paraná, Brazil) and tap water *ad libitum*.

### 2.3. Adaptation to treadmill exercise

Before mating, the females were acclimatized to the treadmill exercise (Motorized Treadmill for Rats, ANSprojects®). During 3 days, 10 min/day, animals underwent the recognition of a motorized horizontal treadmill (5 min, stationary) and running in a low intensity (5 min, 8 m/min, at 0° of inclination) (Kim et al., 2007). Subsequently, during 5 days, 10 min/day, females were

adapted to the treadmill exercise at 16 m/min speed, 0° of inclination, corresponding to 50–60% $VO_2$ max of the animals (previously determined), which is corresponding to a moderate exercise for pregnant rats (Nazıroğlu et al., 2004; Simşek et al., 2005).

### 2.4. Estrous stimulation and mating procedure

During 1 month virgin female rats received wood shavings from the bedding of the male rats for the estrous stimulation. After this period, one male rat was housed individually, and was allowed to mate with 3 virgin female rats for one night. At the beginning of the following morning vaginal smears were taken to detect the presence of sperm, which was the confirmation of the gestational day zero.

### 2.5. Experimental design

After the confirmation of gestational day zero, the females were submitted to intervention:

- Control group (n = 8): sedentary, without exercise.
- Exercise group (n = 8): treadmill exercise, 16 m/min, 0° inclination, 45 min/day, 5 days/week (in a total of 15 days), during 21 gestational days.

On the 3rd day after birth, pups were euthanized by decapitation and their hearts were removed for the achievement of the primary culture of cardiomyocytes. Birth usually occurred at night with 10–12 pups being born, and the hearts of all the neonates were used to obtain a pool of cardiomyocytes. A simplified schedule of the acclimatization of the animals until the achievement of the primary culture of cardiomyocytes is described in Fig. 1.

### 2.6. Primary culture of cardiomyocytes

A primary culture was prepared from the hearts of 3 day old Wistar rats and cultured as previously defined (Fu et al., 2005), with some modifications as follows: the hearts were removed, washed with PBS, minced and defragmented with a buffer containing 0.125% pancreatin and 0.3% BSA diluted in 10 mL (g/L): 8.0 NaCl, 2 KCl, 0.5 $Na_2HPO_4$, 1.0 $NaHCO_3$, and 2.0 dextrose (pH 7.2). This homogenate was digested at 37 °C during repeated cycles of 5 min. The supernatant of each cycle was centrifuged (500 g, 5 °C, 5 min) and the pellet was resuspended in 3 mL DMEM containing 10% SFB and 1% penicillin/streptomycin, and placed in a humidified incubator (5% $CO_2$, 37 °C). This process was repeated until the complete defragmentation of all cardiac tissue. Then, the pool of cells containing fibroblasts and cardiomyocytes was plated in 75 $cm^2$ bottle culture, for fibroblast adhesion. Finally, the cellular suspension containing cardiomyocytes was aspired, centrifuged and plated on culture plates (Falcon, EUA) previously treated with gelatin (0.1% in PBS). The culture was treated with DOX (0.1, 0.5 or 1.0 μM) during 24 or 48 h and the suitable confluence was daily checked for readiness to proceed to the specific analysis.

### 2.7. Trypan blue (TB) exclusion assay

Cell viability was measured by TB exclusion test, as previously described (Robichová and Slamenová, 2002) with some modifications. TB staining is a used method to identify viable cells, which have intact membranes and can effectively exclude the dye, whereas dead cells with compromised membranes become stained. In brief, cardiomyocytes treated during 24 or 48 h with DOX (0.1, 0.5 or 1.0 μM) were washed with PBS, trypsinized, centrifuged and resuspended in PBS. An aliquot of this cellular suspension was stained with TB dye (0.4%), and the number of viable (uncolored) and dead (colored) cells was counted in Automated Cell Counter (Countess™, Thermo Fisher Scientific). The ratio of [(viable cells/total cells) x 100] results in the percentage of viable cells.

### 2.8. Assessment of apoptosis by flow cytometric analysis

Annexin V-PE was used together with a vital dye, 7-AAD, to distinguish apoptotic (Annexin V-PE positive, 7-AAD negative) from necrotic (Annexin V-PE positive, 7-AAD positive) cells, according to the manufacturer's instructions. Cardiomyocytes treated during 24 or 48 h with DOX (0.1, 0.5 or 1.0 μM) were washed with PBS, trypsinized, centrifuged (1500 rpm, 5 °C, 10 min) and resuspended in 100 μL of binding buffer with 3 μL Annexin V-PE and 3 μL of 7-AAD. Cells were mixed gently and incubated for 15 min in the dark and at room temperature. After incubation, 200 μL of binding buffer was added and mixed for immediate analysis. Data were collected and analyzed by a FACS Calibur flow cytometer with CellQuest software, with a maximum of 5000 events per sample. Fluorescence was measured and the percentage of viable, early and late apoptotic, and necrotic cells was determined.

### 2.9. Comet assay

The alkaline comet assay was performed as previously described (Hartmann and Speit, 1997; Singh et al., 1988) with minor modifications. Cardiomyocytes treated during 24 or 48 h

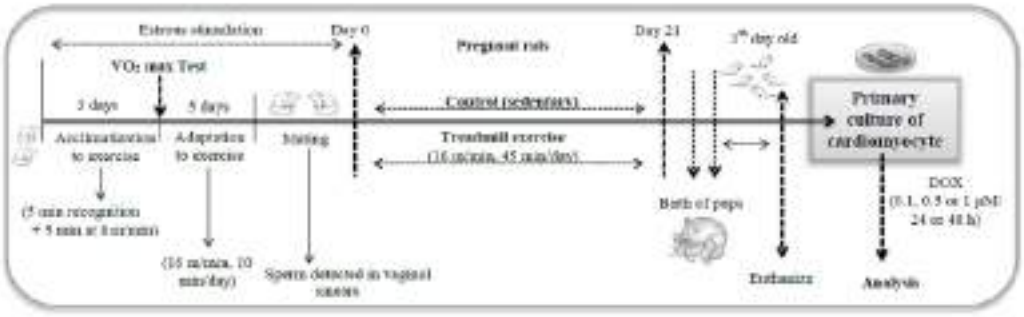


Fig. 1. Experimental design and treatment schedule.

with DOX (0.1, 0.5 or 1.0 μM) were washed with ice-cold PBS, trypsinized, resuspended in complete medium, centrifuged and resuspended again in 200 μL ice-cold PBS. Then, 30 μL of cell suspension was dissolved in 0.75% LMP agarose and immediately spread onto a glass microscope slide pre-coated with a layer of 1% NMP agarose. The slides were then incubated in ice-cold lysis solution at 4 °C for at least 1 day in order to remove cellular proteins and membranes, leaving the DNA as 'nucleoids'.

In the modified comet assay, slides were removed from the lysis solution, washed in enzyme buffer and incubated with 100 μL FPG or EndoIII enzymes (300 mU per gel; 45 min 37 °C). After, the slides were placed on a horizontal electrophoresis unit containing freshly made alkaline buffer, which covered the slides for 20 min at 4 °C in order to allow unwinding of DNA and expression of alkali-labile sites. Subsequently, an electric current (300 mA; 25 V; 0.9 V/cm) was applied for 20 min to allow DNA migration. All the steps listed above were performed in the dark in order to prevent additional DNA damage. Slides were then neutralized and stained according to a silver-staining protocol (Nadin et al., 2001). After drying, one hundred cells (from each of two replicate slides) were analyzed visually with an optical microscope, and scored according to tail length into five classes, assigned as class 0: undamaged, without a tail, to class 4: comets with no head, almost all DNA in tail. A damage index (DI) is an arbitrary score calculated for cells in different damage classes, which are visually scored by measuring DNA migration length and the amount of DNA in the tail. The genotoxic effect of DOX on cardiomyocytes was estimated by damage index (DI) of DNA, which ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) (Hartmann et al., 2003).

### 2.10. Preparation of cardiomyocytes extracts

After treatment of cardiomyocytes during 24 or 48 h with DOX (0.1, 0.5 or 1.0 μM) cells were washed with ice-cold PBS and whole cell protein extracts were obtained by scraping cells in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100) containing a complete Mini protease cocktail inhibitor tablet (Roche Applied Science, QC, Canada). The protein extracts of cardiomyocytes were used for additional analysis.

### 2.11. 2′,7′-Dihydrodichlorofluorescein diacetate ($H_2$DCF-DA) oxidation assay

ROS production was assessed as previously described (LeBel et al., 1992) using the $H_2$DCF-DA marker, which is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent $H_2$DCF that is rapidly oxidized to a highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. Fluorescence intensity was measured in SpectraMax® M2e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California) using excitation and emission wavelengths of 480 and 535 nm, respectively. A calibration curve was performed with standard DCF (1 mM) and the levels of ROS were calculated as nmol DCF formed/mg protein.

### 2.12. Superoxide dismutase (SOD) activity

SOD activity was evaluated by quantifying the inhibition of superoxide-dependent autoxidation of epinephrine, verifying the absorbance of samples at 480 nm (Misra and Fridovich, 1972). The inhibition of autoxidation of epinephrine occurs in the presence of SOD, the activity of which can be then indirectly assayed spectrophotometrically in a SpectraMax® M2e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California). One SOD unit is defined as the amount of SOD necessary to inhibit 50% of epinephrine autoxidation and the specific activity is reported as SOD Units/mg protein.

### 2.13. Catalase (CAT) activity

CAT activity was assayed according to the previously described method (Aebi, 1984), based on the disappearance of $H_2O_2$ at 240 nm. The absorbance was recorded in a kinetic protocol using a SpectraMax® M2e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California). One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the activity is calculated as CAT Units/mg protein.

### 2.14. Western blot analysis

A Western blot analysis was performed essentially as previously described (Towbin et al., 1979). For this, 25 μg of cardiomyocyte

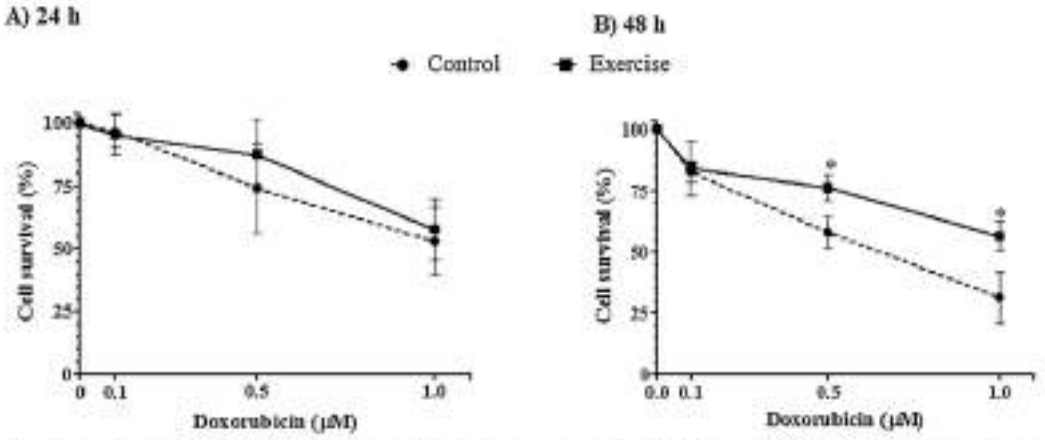


Fig. 2. Effects of exercise during pregnancy on neonatal cardiomyocyte viability. Cells were treated with DOX (0.1, 0.5 or 1.0 μM) for 24 h (A) or 48 h (B). Values are mean ± SD (n = 8). The symbol * indicates $p < 0.05$ from control group, by One-Way ANOVA, post-hoc Tukey.

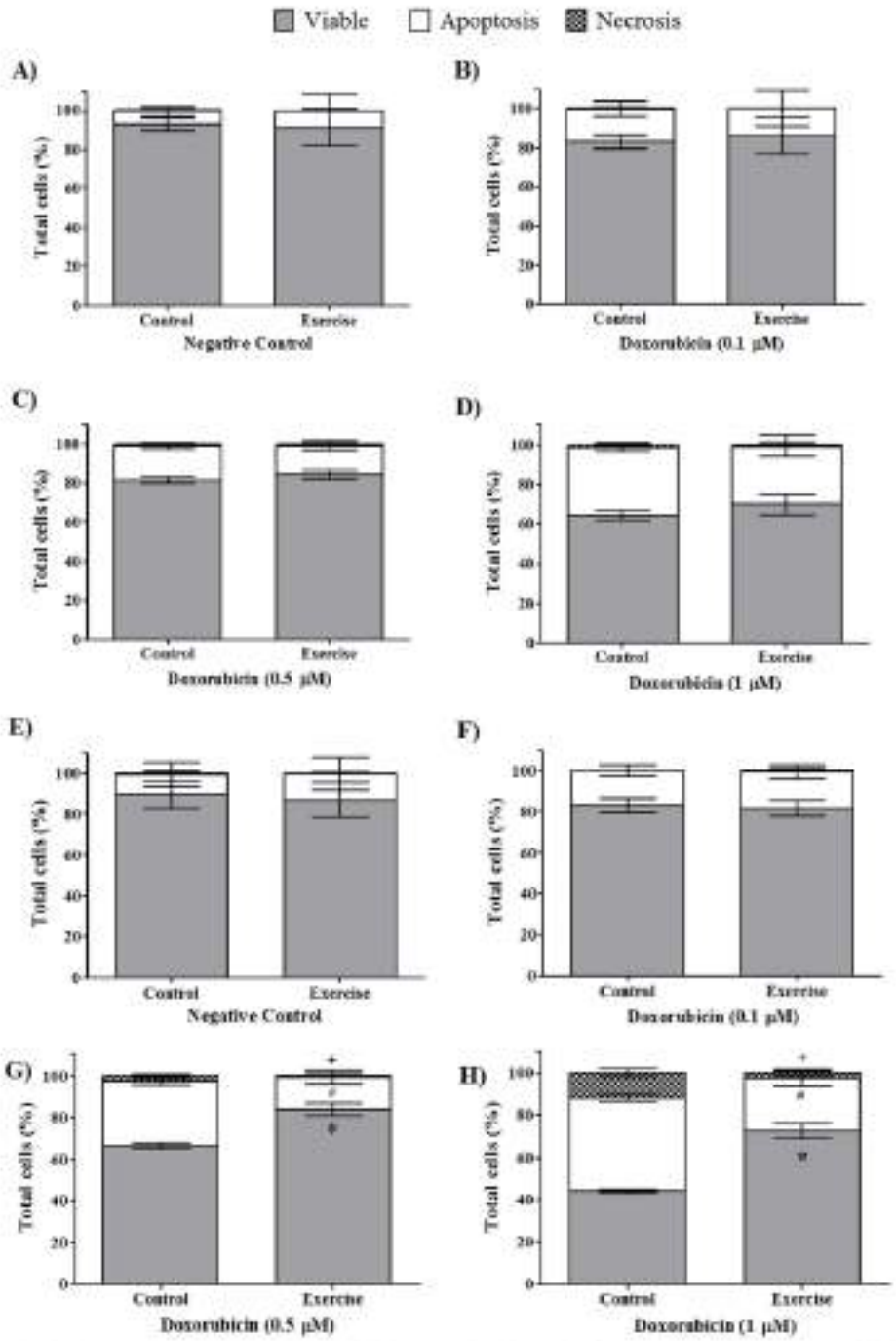


Fig. 5. Effects of exercise during pregnancy on neonatal cardiomyocyte viability, apoptosis, and necrosis by flow cytometric analysis. Cells were treated 24 h (A–D) or 48 h (E–H) with DOX (0.1, 0.5 or 1.0 μM). Negative control = cells without DOX. Values are mean ± SD (n = 8). The symbol * indicates viable, # apoptotic and + necrotic cells, with $p < 0.05$ from control group, by One-Way ANOVA, post-hoc Tukey.

proteins were separated by electrophoresis on a 12% SDS-PAGE and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad®), blocked with 5% BSA for 2 h, and incubated overnight at 4 °C with the primary antibodies anti-sirt6 (ab62739) and anti-actin (C-2) (sc-8432), both at 1:500. The blot was washed with TTBS (Tris-buffered saline (TBS) pH7.5, plus 0.05% Tween-20) and incubated for 2 h in TTBS solution containing the secondary antibody peroxidase conjugated (1:3000). The blot was then developed using a chemiluminescence ECL kit. Immunoblots were quantified by scanning the films with a DNR Bio-Imaging Systems scanner (MF ChemiBis2.0®) and determining optical densities with ImageJ 1.48v software (Wayne Rasband, National Institutes of Health, USA).

### 2.15. Protein quantification

The protein concentration of the cardiomyocyte protein extracts was determined with the method previously described (Lowry et al., 1951) using serum bovine albumin as the standard.

### 2.16. Statistical analysis

The normal distribution of variables was tested with the Kolmogorov-Smirnov normality test and the homogeneity of variances with Levene's test. Data were compared between groups by One-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test. Correlations were performed by Pearson's correlation coefficient. The Statistical Package for the Social Sciences (SPSS, version 16.0) was used for all analyses, and the GraphPad Prism 5.03 program (GraphPad Software, San Diego, CA) was used as a computational tool for graph edition. Data were expressed as mean ± SD, and a p value < 0.05 was considered significant.

## 3. Results

### 3.1. Exercise protects neonatal cardiomyocytes from DOX-induced death

Viability of the neonatal cardiomyocytes by TB exclusion test, is represented in Fig. 2. A concentration-dependent cell death induced by DOX, both at 24 and 48 h after treatment was observed. Exercise of mothers during pregnancy protected the cardiomyocytes of progeny from death induced with 0.5 and 1.0 μM DOX after 48 h (Fig. 2B) compared with neonatal cardiomyocytes from the control group.

### 3.2. DOX-induced apoptosis and necrosis are reduced in neonatal cardiomyocytes from exercised mothers during pregnancy

In an attempt to understand the main mechanism of DOX-induced death in this model, and the effects of exercise, neonatal cardiomyocytes treated with DOX were analyzed by flow cytometry (Fig. 3). Similarly to the viability results, it was observed that there was a concentration-dependent increase in cardiomyocytes death (24 and 48 h after DOX treatment), and that exercise did not protect against DOX-induced cell death 24 h after treatment (Fig. 3A–D). However, neonatal cardiomyocytes from mothers exercised during pregnancy exhibited a protection against DOX-induced death 48 h after DOX (0.5 and 1.0 μM) treatment, with an increase in total viable cells and a decrease of apoptotic and necrotic cells (Fig. 3E–H). These results demonstrate that apoptosis is the main mechanism of DOX-induced death in cardiomyocytes of progeny and it is significantly reduced by the exercise during pregnancy.

### 3.3. Exercise protects neonatal cardiomyocytes against ROS increase

As can be seen in Fig. 4A, neonatal cardiomyocytes exposed to DOX (0.5 and 1.0 μM) showed an increase in ROS production in relation to cells not exposed to the drug (negative control). Also, exercise during pregnancy did not protect neonatal cardiomyocytes 24 h after DOX treatment. An increase in ROS production induced by DOX was also observed 48 h after treatment of neonatal cardiomyocytes from sedentary mothers with 0.5 and 1.0 μM DOX (Fig. 4B). However, the exercise of mothers during pregnancy significantly protected neonatal cardiomyocytes against the ROS increase 48 h after DOX treatment. In addition, Pearson's analysis demonstrates an inverse correlation between cell viability and ROS production in neonatal cardiomyocytes both at 24 and 48 h ($r = -0.799$, $p < 0.0001$ and $r = -0.776$, $p < 0.001$), respectively.

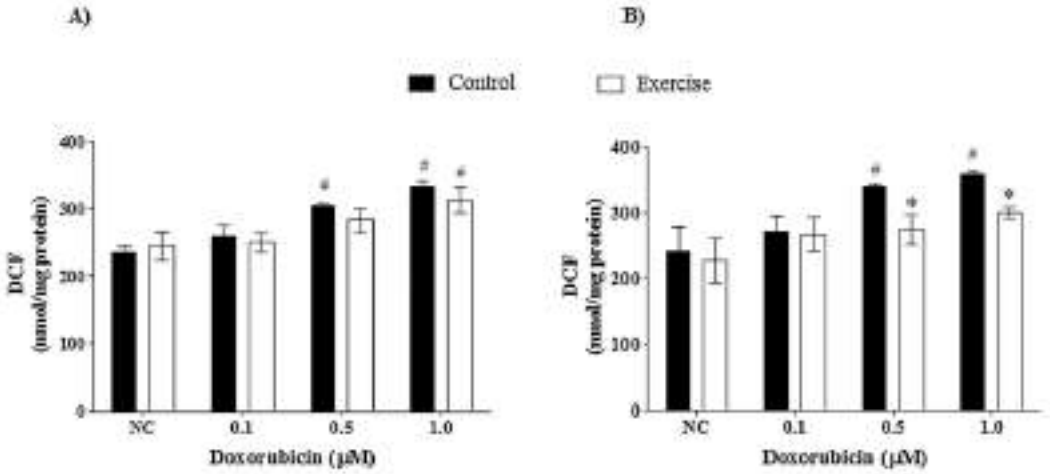


Fig. 4. Effects of exercise during pregnancy on ROS production in neonatal cardiomyocytes. Cells were treated with DOX (0.1, 0.5 or 1.0 μM) for 24 h (A) or 48 h (B). Values are mean ± SD (n = 8). The symbol * indicates $p < 0.05$ from control group and # indicates $p < 0.05$ from negative control (NC – cells without DOX), by One-Way ANOVA, post-hoc Tukey.

Importantly, there was a significant direct correlation between ROS production and apoptosis ($r = 0.658$, $p < 0.0001$, $r = 0.693$, $p < 0.0001$) observed both at 24 and 48 h after DOX treatment, respectively.

### 3.4. Oxidative DNA damage response is induced in cardiomyocytes of progeny by exercise of mothers during pregnancy

Subsequently, in order to verify the DOX effects on the DNA integrity of neonatal cardiomyocytes, an analysis of DNA damage by alkaline Comet assay was carried out. This assay detects primary (repairable) DNA single and double-strand breaks and alkali-labile sites (Collins et al., 1993; Dušinská and Collins, 1996). It can be seen in the representative images of cardiomyocytes from sedentary mothers that DOX induced a concentration-dependent increase in DNA damage (Fig. 5A–D). The analysis of DNA damage, through a damage index, shows a clear concentration-dependent increase of DOX-induced DNA damage observed in neonatal cardiomyocytes of all groups of mothers (Fig. 6A and B). However, exercise by mothers during pregnancy protected cardiomyocytes of progeny from DOX-induced DNA damage, both at 24 and 48 h after DOX treatment. It is important to mention that exercise was able to decrease DNA damage even in cells not treated with DOX (negative control).

Fig. 6(C–F) shows DOX-induced DNA damage after analysis with the DNA-repair enzymes EndoIII and FPG. These enzymes increase the Comet test specificity and are able to recognize oxidative base damaged and convert it into single-strand breaks (Collins et al., 1993; Hartmann et al., 2003). This result shows the extent of oxidative DNA damage caused by DOX treatment in neonatal cardiomyocytes. This oxidative damage was recognized by EndoIII and FPG and was significantly lower in neonatal cells from exercised mothers in relation to neonatal cells from controls. Moreover, at same form observed in alkaline comet assay, neonatal cardiomyocytes (from exercised mothers) not exposed to DOX showed a decrease in oxidative DNA damage, both at 24 and 48 h after DOX treatment. Importantly, Pearson's analysis demonstrates a direct correlation between EndoIII or FPG activity and ROS production in neonatal cardiomyocytes both at 24 h ($r = 0.750$, $p < 0.0001$ and $r = 0.622$, $p < 0.0001$) and 48 h ($r = 0.774$, $p < 0.001$ and $r = 0.753$, $p < 0.001$) after DOX treatment, for EndoIII and FPG, respectively.

### 3.5. Exercise protects neonatal cardiomyocytes against a decrease in antioxidant defenses levels

In order to understand the possible mechanisms of cardioprotection induced by exercise, we evaluated the activity of antioxidant enzymes SOD and CAT, as shown in Table 1. Treatment with DOX induced a decrease in SOD activity levels of neonatal cardiomyocytes from control mothers compared to the exercised, both at 24 and 48 h after DOX treatment. Also, SOD activity of neonatal cardiomyocytes from the exercised group was not affected by an increase in DOX concentrations. Conversely, DOX induced a decrease in the CAT activity, which was more pronounced in neonatal cardiomyocytes from control mothers. Moreover, exercise during pregnancy caused a significant increase in CAT activity levels of cardiomyocytes in all concentrations of DOX and also in the negative control. In particular, CAT activity showed an inverse correlation with ROS production ($r = -0.791$, $p < 0.0001$ and $r = -0.808$, $p < 0.0001$) and a direct correlation with cell viability ($r = 0.572$, $p < 0.0001$ and $r = 0.716$, $p < 0.0001$) both at 24 and 48 h after DOX treatment, respectively.

### 3.6. Levels of Sirt6 expression are increased in neonatal cardiomyocytes from exercised mothers during pregnancy

Western blotting analysis showed that exercise during pregnancy increases the expression of histone deacetylase Sirt6 in cardiomyocytes of progeny. This effect was also observed in neonatal cells from exercised mothers not exposed to DOX and was higher at 1 μM DOX (increase of 3; 3.4; 3.5 and 4.1-fold compared to neonatal cells from control mothers treated with NC; 0.1; 0.5 and 1.0 μM DOX, respectively) (Fig. 7).

## 4. Discussion

The main adverse effect of DOX is cardiotoxicity, which can cause congestive heart failure in adults at cumulative doses. Children and adolescents are particularly susceptible and there is no safe dose in this population (Kocabaş et al., 2014; Trachtenberg et al., 2011). Among the multiple mechanisms of DOX-induced toxicity, it is established that free radical-induced oxidative stress plays an important role in the pathogenesis of heart failure, leading to apoptosis, necrosis and autophagic cardiomyocyte death (Cascales et al., 2012; Rochette et al., 2015). Here we demonstrated that DOX induced an increase in ROS production in neonatal cardiomyocytes and this effect was correlated with the decrease in

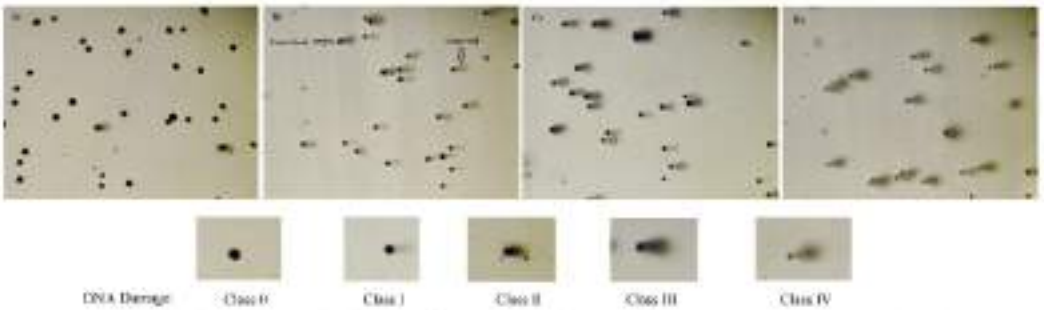


Fig. 5. Representative image of DNA damage in neonatal cardiomyocytes from sedentary mothers. NC – cells without DOX (A), 0.1 μM (B), 0.5 μM (C), and 1.0 μM (D) DOX for 24 h. One hundred cells were scored according to tail length into five classes, assigned as class 0: undamaged, without a tail, to class 4: comets with no head, almost all DNA in tail. Images were represented as damage index (DI) of DNA, which ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). The images were obtained with a digital color camera (Olympus IMS DP72) for microscope. Mag. 10 x.

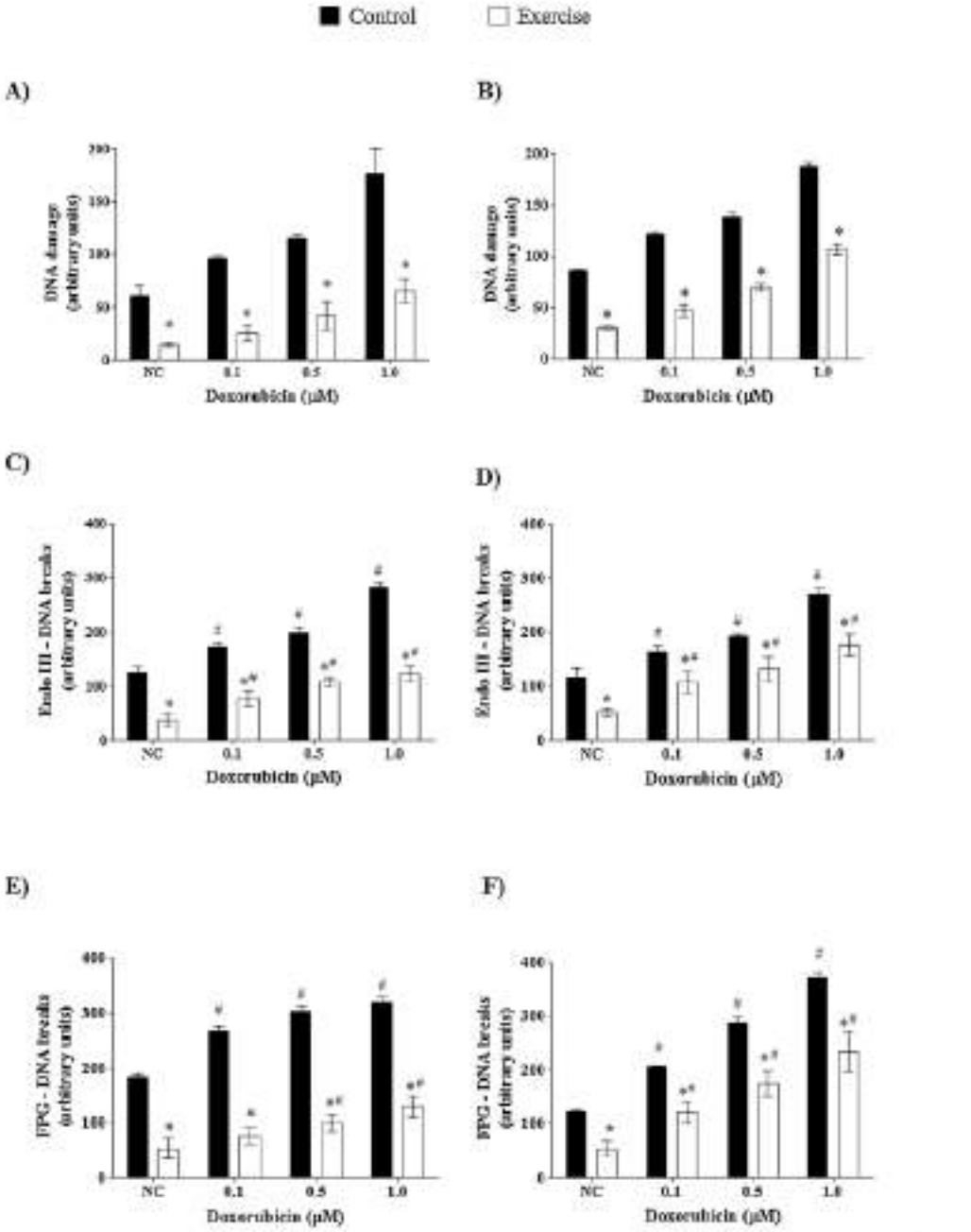


Fig. 6. Effects of exercise during pregnancy on DNA damage of neonatal cardiomyocytes. Cells were treated with DOX (0.1, 0.5 or 1.0 μM) for 24 h (A) or 48 h (B) and DNA damage due to single and double-strand breaks and alkali-labile sites were detected. Effects of exercise on oxidative DNA damage were analyzed with Endo III and FPG enzymes when neonatal cardiomyocytes were treated with DOX (0.1, 0.5 or 1.0 μM) for 24 h (C and E) or 48 h (D and F). These enzymes recognize oxidative base damage and convert it into single strand breaks. Values are mean ± SD (n = 8). The symbol * indicates $p < 0.05$ from control group, and # indicates $p < 0.05$ from negative control (NC – cells without DOX), by One-Way ANOVA, post-hoc Tukey.

**Table 1**
Effects of exercise during pregnancy on SOD and CAT activity levels of neonatal cardiomyocytes.

| DOX (μM) treatment | Control SOD (U/mg protein) | Exercise | Control | Exercise |
|---|---|---|---|---|
| | 24 h | | 48 h | |
| NC | 4.81 ± 0.54 | 5.43 ± 1.03 | 4.48 ± 0.30 | 5.28 ± 1.12 |
| 0.1 | 3.90 ± 0.68 | 5.58 ± 1.11* | 3.98 ± 0.08# | 5.60 ± 0.31* |
| 0.5 | 3.23 ± 0.72# | 5.32 ± 0.58* | 3.60 ± 0.26# | 5.21 ± 0.92* |
| 1.0 | 2.83 ± 0.40# | 5.02 ± 1.00* | 2.53 ± 0.93# | 5.52 ± 1.61* |
| DOX (μM) treatment | CAT (U/mg protein) | | | |
| | 24 h | | 48 h | |
| NC | 12.15 ± 2.16 | 31.36 ± 4.52*# | 12.30 ± 0.93 | 32.48 ± 8.37*# |
| 0.1 | 6.16 ± 0.81# | 17.25 ± 3.64*# | 7.85 ± 1.03# | 14.32 ± 2.03*# |
| 0.5 | 4.64 ± 0.57# | 10.89 ± 3.88*# | 5.35 ± 0.62# | 10.53 ± 4.17*# |
| 1.0 | 2.62 ± 0.20# | 7.72 ± 1.03*# | 2.08 ± 1.58# | 8.23 ± 2.45*# |

Cells were treated with DOX (0.1, 0.5 or 1.0 μM) during 24 or 48 h. Values are mean ± SD (n = 8). The symbol * indicates $p < 0.05$ from control group, and # indicates $p < 0.05$ from negative control (NC – cells without DOX), by One-Way ANOVA, post-hoc Tukey.

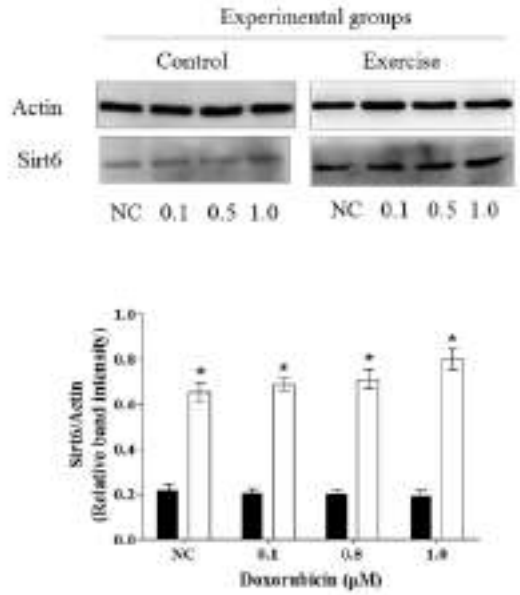


Fig. 7. Effects of exercise during pregnancy on Sirt6 expression in neonatal cardiomyocytes. Cells were treated with DOX (0.1, 0.5 or 1.0 μM) for 48 h. Bar graph corresponds to mean ± SD of the quantification values of Sirt6/Actin ratio from all samples. The symbol * indicates $p < 0.05$ from control group, by One-Way ANOVA, post-hoc Tukey.

cell viability. Apoptosis was the main mechanism of DOX-induced cardiomyocyte death, which was correlated with the increase in ROS production and consistent with the mechanisms of DOX toxicity.

Despite searches for adjuvant therapies, an effective treatment to counteract the cardiotoxic effects of DOX is yet to be discovered. It is known that exercise training provides myocardial protection against many cardiac insults. In fact, exercise can constitute an excellent tool either to prevent and/or to treat several diseases, providing enhanced resistance to the cardiac muscle tissue (Ascensão et al., 2003; Ferreira et al., 2016; Yamamoto et al., 2016). In experimental models, when exercise is performed before (Ascensão et al., 2011a; Kavazis et al., 2014) and during DOX treatment (Marques-Aleixo et al., 2015) it seems to attenuate cardiac dysfunction. However, because DOX is used in patients undergoing cancer chemotherapy who experience severe fatigue and display considerable exercise intolerance, the intensity and duration of exercise training sessions can constitute a limiting factor, and an alternative model for using exercise is interesting.

Here we investigated for the first time a model of intervention with exercise conducted during pregnancy, evaluating its possible protective effects on the cells of progeny, which were exposed to DOX. Our results indicated that neonatal cardiomyocytes from mothers exercised during pregnancy exhibited a protection against DOX-induced apoptotic and necrotic cell death, showing an increase in cell viability correlated with the decrease in ROS production. The cardioprotection induced by exercise can be attributed to the activation and up-regulation of antioxidant enzymes. In this line, after DOX treatment the neonatal cardiomyocytes from exercised mothers exhibited levels of SOD and CAT activities higher than cardiomyocytes from sedentary mothers. Moreover, exercise during pregnancy induced an increase in CAT activity also in cardiomyocytes of progeny untreated with DOX. These effects of exercise on antioxidant status were correlated with the decrease in ROS production and also with cardiomyocytes death, assigning to exercise performed during pregnancy an important protective effect against DOX-induced cardiotoxicity. These data can also be linked to a phenomenon usually referred to as an exercise-induced cross-tolerance, where the exercise training has been associated to an up-regulation of the heart antioxidant system and mitochondrial function, reduction of the lipid peroxidation by-products formation and induction of heat shock protein (HSP) overexpression after stress stimuli (Kavazis et al., 2010; Koltai et al., 2010a; Lee et al., 2012; Powers et al., 2006, 2014; Venditti et al., 2015). Moreover, it has been demonstrated that exercise is able to improve the mitochondrial respiratory function, decreasing the doxorubicin-induced toxicity in rats (Ascensão et al., 2011a, 2011b, 2005b), in agreement with the essential role of mitochondria in the pathogenesis of DOX-induced cardiotoxicity. Although in this model the exercise has been made by mothers, an intergenerational effect of maternal exercise on mitochondrial function in cardiomyocytes of progeny may be possible.

An important question of this work is regarding the effects of exercise on DNA integrity. Surprisingly, exercise during pregnancy was able to decrease DNA strand-breaks due to oxidative stress, measured by FPG and EndoIII enzymes, in the cardiomyocytes of progeny. The reduction, induced by exercise, in oxidative DNA strand-breaks was also observed in the absence of DOX treatment, indicating that the mechanisms of DNA repair may be induced by

exercise, repairing basal levels of DNA damage and possibly modulating the cell repair machinery for additional insults. In line with this, exercise during pregnancy induced an important increase in the expression of Sirt6 in neonatal cardiomyocytes. Sirt6 is a nuclear HDAC that removes acetylation from lysine residues within histones and, together with other sirtuins, has received prominence for its involvement in genome stability, the chromatin remodeling process and DNA damage response (Cardus et al., 2013; Tennen and Chua, 2011). Similarly to other class III HDACs, Sirt6 is dependent on $NAD^+$, a classical coenzyme with a well-established role in cellular redox reactions. Thus, the increase in the NAD:NADH ratio, which can be induced by exercise can increase the expression and activity of this sirtuin (Koltai et al., 2010b; Winnik et al., 2015). It has been demonstrated that activity of Sirt6 is reduced together with the intracellular $NAD^+$ level during cardiac hypertrophy, indicating a relationship between $NAD^+$ and Sirt6 in the heart (Cai et al., 2012). $NAD^+$ levels increase during exercise, acting as a metabolic sensor and leading to Sirt6 up-regulation (Morris, 2013) and supporting the Sirt6 activation observed in neonatal cardiomyocytes from exercised mothers.

An important function of Sirt6 is related to genome stability and chromatin remodeling (Mao et al., 2011; Toiber et al., 2013). Through oxidative stress situations, such as those that are DOX-induced, Sirt6 is recruited to sites of DNA double-strand breaks, where it stimulates DNA repair through both non-homologous end-joining and homologous recombination. Consequently, Sirt6 binds to and mono-ADP-ribosylates PARP-1, leading to increased PARP poly-ADP-ribosylase activity and DNA repair (Mao et al., 2011). Also, Sirt6 is a defense protein and cells try to activate defense pathways to survive, which occurs under stressful events such as hypoxic damage to the heart (Maksin-Matveev et al., 2015), cardiac hypertrophy (Cai et al., 2012), premature chondrocytes senescence (Nagai et al., 2015) and, in our results, DOX-induced DNA damage.

Other ways are involved in repair provided by Sirt6, such as deacetylation of C-terminal binding protein interacting protein (CtIP), which is essential for DNA end-resection during homologous recombination evoked by DNA double-strand breaks (Kaidi et al., 2010). In the DNA break sites Sirt6 also recruits SNF2H, an ATP-dependent chromatin remodeler, to open up condensed chromatin and providing additional tools in genomic stability (Toiber et al., 2013).

Regarding the effects of exercise on the phenotype observed in progeny, it was recently suggested that exercise acts as a regulator of epigenetic modifications that are also involved in the pathogenesis of cardiovascular diseases (Zimmer and Bloch, 2015). Evidences have shown that exercise can induce epigenetic modifications implicated in the regulation of cardiovascular disease-associated genes, with consequent regulation of pro-inflammatory cytokines; vascular smooth muscle fiber differentiation; endothelial function; and changes in cardiomyocytes myosin heavy chain expression (Alexander and Owens, 2012; Gielen et al., 2011; Nakajima et al., 2010; Soci et al., 2016). Moreover, the epigenetic modifications at chromatin level are controlled by a set of specialized enzymes (e.g., the HDACs and histone acetyltransferases), metabolite availability, and signaling pathways (Migicovsky and Kovalchuk, 2011; Waddington, 2012). Many of these enzymes are dependent on changes in the levels of metabolites, such as oxygen, tricarboxylic acid cycle intermediates, and $NAD^+$ levels, being susceptible to changes induced by exercise in a tissue-dependent manner (Fan et al., 2015; Pareja-Galeano et al., 2014). Therefore, exercise-induced metabolic alterations can influence epigenetic modifications by changing local concentrations of key metabolites. If the exercise-induced epigenetic modifications can be transmitted to progeny and cause modulation of heart gene expression is an unsolved question. However, as many genes can be epigenetically modified by exercise, or exercise-induced metabolic alterations, it can be hypothesized that exercise during pregnancy could mediate beneficial responses, with cardioprotective effects in progeny (Donovan and Miller, 2011; Sharples et al., 2016).

Therefore, this work investigated the effects of exercise on DOX-toxicity by a novel model of study. Here we demonstrate for the first time that exercise during pregnancy is able to protect the cardiomyocytes of progeny against DOX-toxicity, and this cardioprotection was mainly related to the inhibition of apoptotic cell death. The findings of this work indicate that neonatal cardiomyocytes from exercised mothers during pregnancy are notably more resistant to DOX-induced toxicity, which occurs by the modulation of the redox status, by decreasing ROS production and DNA oxidative damage, an increase in antioxidant activity, and by the regulation of the chromatin remodeling and DNA repair signaling protein – Sirt6. This result could be related to an intergenerational cardioprotective effect, denoting an important involvement of the maternal environment in answers to stressful agents of progeny throughout life. However, additional studies are necessary to clarify the role of epigenetics in the cardioprotective phenotype awarded by exercise observed in progeny.

## Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

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**5. CAPÍTULO 2: ARTIGO 2:** Maternal resveratrol supplementation during pregnancy protects neonatal heart against Doxorubicin-induced cardiotoxicity.

**Maternal resveratrol supplementation during pregnancy protects neonatal heart against Doxorubicin-induced cardiotoxicity.**

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# Maternal resveratrol supplementation during pregnancy protects neonatal heart against Doxorubicin-induced cardiotoxicity.

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**Abbreviations**

7-AAD, 7-Amino-Actinomycin; AMP/ATP, AMP to ATP ratio; AMPK, AMP-activated protein kinase; Annexin V-PE, Annexin V-Phycoerythrin; BSA, bovine serum albumin; CAT, catalase; DCF, 2′-7′-dichorofluorescein; DCF-DA, 2′-7′-dichorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DOX, doxorubicin; DTNB, 5-5-dithio-bis (2-nitrobenzoic acid); EndoIII, endonuclease III; FBS, fetal bovine serum; FPG, formamidopyrimidine DNA-glycosylase; HDAC, histone deacetylase; HMP, high melting-point; LMP, low melting-point; $NAD^+$, nicotinamide adenine dinucleotide; PARP, poly-ADP-ribose polymerase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; Sirt6, sirtuin6; SOD, superoxide dismutase; TB, trypan blue.

**Abstract**

Treatment of cancer with doxorubicin (DOX) is limited by its dose-dependent cardiotoxicity, increasing the demand for more reasonable therapeutics. Considering that bioactive components of diet can influence epigenetic factors and health of future generations, this research investigated the effects of resveratrol supplementation during pregnancy on DOX-induced cardiotoxicity in heart of neonatal rats. For this, rats were divided in control and resveratrol groups and pre-treated during gestational days. Hearts of newborns were used to obtain the cardiomyocytes culture that was treated with DOX. The results showed that maternal resveratrol supplementation induced an increase in neonatal cardiomyocytes viability and a decrease in DOX-induced apoptotic and necrotic cell death, which were correlated to the decrease in ROS production and increase in antioxidant defenses. Resveratrol considerably protected neonatal cardiomyocytes from DOX-induced DNA damage, demonstrating a reduction in the oxidative DNA breaks. Notably, resveratrol supplementation induced an increase in expression of Sirt6 in neonatal cardiomyocytes exposed to DOX. These results demonstrate that resveratrol supplementation during pregnancy has a cardioprotective effect on offspring heart against DOX-induced toxicity, in part due its antioxidant function, and mainly related to Sirt6 activation and DNA damage repair, promoting the genome stability.

**Key-words**: *resveratrol; pregnancy; doxorubicin; cardiomyocytes; Sirt6; oxidative stress.*

# 1. Introduction

In the last decades the survival of cancers has enhanced due to the better screening and the advance in therapy. The anthracycline antibiotics are an important group of antitumor drugs widely used in cancer chemotherapy. Among these, doxorubicin (DOX) is a broad spectrum chemotherapeutic agent and it is one of the most widely used therapy for treating a variety of adult and pediatric solid tumors and leukemias (Cortés-Funes and Coronado, 2007; Minotti et al., 2004). The main cytotoxic action of DOX in cancer cells involves DNA intercalation, topoisomerase inhibition and production of free radicals to attack DNA, which compromises the replication and transcription process (Ferrans, 1978; Gewirtz, 1999). However, the clinical effectiveness of DOX is limited by its side effects, with strong cardiotoxicity leading to dose-dependent cardiomyopathy (Grenier and Lipshultz, 1998; Volkova and Russell, 2011), and the development of multidrug resistance (Kaye and Merry, 1985; Villar et al., 2014).

DOX can interfere with many different intracellular processes, so it has been described that molecular etiologies which give rise to acute and chronic cardiotoxicity are multiple and unsolved. A variety of pathways has been proposed, but the most commonly accepted mechanism is the iron-mediated formation of reactive oxygen species (ROS), which promotes myocardial oxidative stress (Lebrecht et al., 2007; Šimůnek et al., 2009). This ROS generation can cause damage to lipid membranes and nucleic acids, which together with reduced levels of antioxidants and sulfhydryl groups leads to the initiation of apoptosis in cell (Liu et al., 2007; Wang et al., 2001). Moreover, DOX can also induce DNA damage, inhibit DNA and protein synthesis, promote myofibril degeneration, inhibit transcription of specific genes and induce cardiomyocyte apoptosis via caspase-3 dependent pathway (Gratia et al., 2012; Montaigne et al., 2012; Tokarska-Schlattner et al., 2006).

Elucidation of the mechanism of DOX-induced cardiomyopathy and its prevention is still unsolved and the most effective method to prevent its cardiotoxicity is a dose-limiting approach that may however compromise its chemotherapeutic properties (Bristow et al., 1981; Jones et al., 2006; Preobrazhenskaya et al., 2006). The present challenge is to design a cardioprotective protocol for both short and long-term effects of DOX without hampering the antitumor activity of the drug. Many preventive and therapeutic strategies have been explored to counteract DOX toxicity and dysfunction, such as antioxidant supplementation and exercise training protocols.

In line with this, studies suggest that Mediterranean diets, characterized by consumption of a large quantity of plant-derived foods, cereals, and fish, low intake of meat and dairy products, daily intake of olive oil and nuts as main source of fat, and moderate intake of wine (especially red wine) during meals, are associated with reduced risk of cardiovascular disease (Grosso et al., 2015; Perona et al., 2010; Tektonidis et al., 2015). Particularly, the cardioprotective effect of wine has been attributed to antioxidants present in its polyphenol fraction (Das et al., 1999; Sato et al., 2002), in view of grapes contain many antioxidants, including resveratrol, epicatechin, catechin and proanthocyanidins. Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a non-flavonoid polyphenolic compound belonging to the stilbene group, has recently gained attention with regard to its potential role in the protection against metabolic and cardiovascular diseases (Catalgol et al., 2012; Das and Maulik, 2006). The cardiovascular benefits of resveratrol may be due its direct or indirect effects in biological systems, such as, it prevents platelet aggregation in vitro (Wang et al., 2002); increases expression of both endothelial and inducible nitric oxide synthase and relaxes isolated arteries and rat aortic rings (Das et al., 2005; Jäger and Nguyen-Duong, 1999; Naderali et al., 2000); exerts antioxidant effects, preventing LDL oxidation in vitro by chelating copper, as well as by directly scavenging free radicals (Frankel et al., 1993); decrease total cholesterol concentration in

hypercholesterolaemic models (Kollár et al., 2000); in addition to its phytoestrogenic action in some systems (Gehm et al., 1997).

Recently, it has been demonstrated that the heritable information is not only transmitted to offspring through DNA sequence. The new area of epigenetic emerges to explain the heritable changes in gene expression not related to changes in DNA sequence (Daxinger and Whitelaw, 2012; Migicovsky and Kovalchuk, 2011; Whitelaw, 2008). Some works in the field of epigenetics has proposed that the inter- and transgenerational transmission of physical, behavioral, and emotional traits in mammals is influenced by a variety of (epigenetic) factors, which can be modulated by chemicals agents, nutritional components of diet, environmental stimulus or toxicants, lifestyle, as well as by aging (Anway et al., 2006; Bollati et al., 2009; Roth et al., 2009; Szyf et al., 2008; Waterland et al., 2006). Particularly, it has been demonstrated that nutritional components of diet can influence epigenetic mechanisms modulating the epigenome, with persistent effects whose can be transmitted to offspring or subsequent generations (Burdge et al., 2007; Dolinoy et al., 2007, 2006; Lillycrop, 2011; Waterland et al., 2006).

Among the most extensively studied epigenetic modifications, highlights the histone acetylation, which is mediated by histone acetyl transferases (HATs) and histone deacetylases (HDACs) enzymes, whose are critically involved in regulating affinity binding between the histones and DNA backbone (Choi and Howe, 2009; Kouzarides, 2007). HDACs class III comprises seven members of sirtuins (Sirt1 to Sirt7) dependent on nicotinamide adenine dinucleotide ($NAD^+$), that modulate lysine acetylation in a dynamic fashion, playing a relevant role in the pathogenesis of various diseases, aging, oxidative stress, cell metabolism, and apoptosis (D'Onofrio et al., 2015; Morris, 2013; Radak et al., 2013; Sebastián and Mostoslavsky, 2015; Wątroba and Szukiewicz, 2015). Particularly, it has been recently demonstrated that Sirt6, a nuclear HDAC with high levels of expression in mouse muscle, brain and heart (Liszt et al.,

2005) is associated with prolonged life expectancy and has an important role in resistance to DNA damage and suppression of genomic instability (Mao et al., 2011; Mostoslavsky et al., 2006; Tennen et al., 2010). Due these features Sirt6 constitutes a potential target to study genome stability and epigenetic regulation in mammals.

Therefore, in view that the components of diet can induce epigenetic modifications and that resveratrol is a bioactive compound that can provide beneficial effects on health and cardiovascular diseases, this work was carried out to investigate the effects of maternal resveratrol supplementation during pregnancy on DOX-induced cardiotoxicity on heart of progeny, examining the possible resveratrol cardioprotective effects on heart defense programming.

## 2. Methods

### *2.1. Chemicals*

DMEM, PBS, FBS and penicillin/streptomycin were obtained from Gibco-BRL (Grand Island, NY, USA). LMP agarose, HMP agarose, hydrogen peroxide, DCF-DA, DCF, catalase, TB, epinephrine, BSA, DTNB and pancreatin were purchased from Sigma (St. Louis, MO, USA). *Trans*-Resveratrol was obtained Cayman Chemical Company (USA). Primary antibody anti-Sirt6 was purchased from Abcam (UK). All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V-PE and 7-AAD were purchased from BD Biosciences (San Diego, CA). FPG and EndoIII were obtained from BioLabs (New England, USA). All other reagents were of analytical grade and purchased from local commercial suppliers.

*2.2. Ethical approval and animals*

The research was performed in accordance with the Brazilian Guidelines for the Care and Use of Animals for Scientific and Didactic Purposes (DOU 27/5/13, MCTI, p.7), and all procedures outlined were approved by the Research Ethics Committee of the UFCSPA (CEUA, number 106/13-182/13).

Female and male albino Wistar rats (aged 4 weeks, weighing 70-100 g) from the Center of the Reproduction of Laboratory Animals of the UFCSPA, were kept under a day/night cycle (lights on 7:00 am to 7:00 pm), room temperature 21ºC ± 1, and 50% ± 5 relative humidity. The animals received throughout the experiment a standard pellet diet (Nuvital CR1®, Paraná, Brazil) and tap water *ad libitum*.

*2.3. Estrous stimulation and mating procedure*

Virgin female rats received wood shavings of the male box for the estrous stimulation. After the first estrous period, one male rat was housed individually for to mate with 3 virgin female rats for one night. At subsequent morning the females were separated and vaginal smears taken to detect the presence of sperm, which was the confirmation of the gestational day zero.

*2.4. Experimental design*

After the confirmation of gestational day zero, the females were assigned in the following groups: 1) Control (n=8): without supplementation with resveratrol, and 2) Resveratrol (n=8): resveratrol 2.5 mg/Kg body weight (dispersed in saline with 0.05% of Tween 80) by gavage. Once a day, 5 days/week, during 21 gestational days.

After birth, pups at 3$^{th}$ life's day were euthanized by decapitation and hearts were removed for achievement of the primary culture of cardiomyocytes. Birth usually occurred at

night with 10-12 pups being born, and hearts of all neonates were used to obtain a pool of cardiomyocytes. A simplified schedule is illustrated in **Figure 1**.

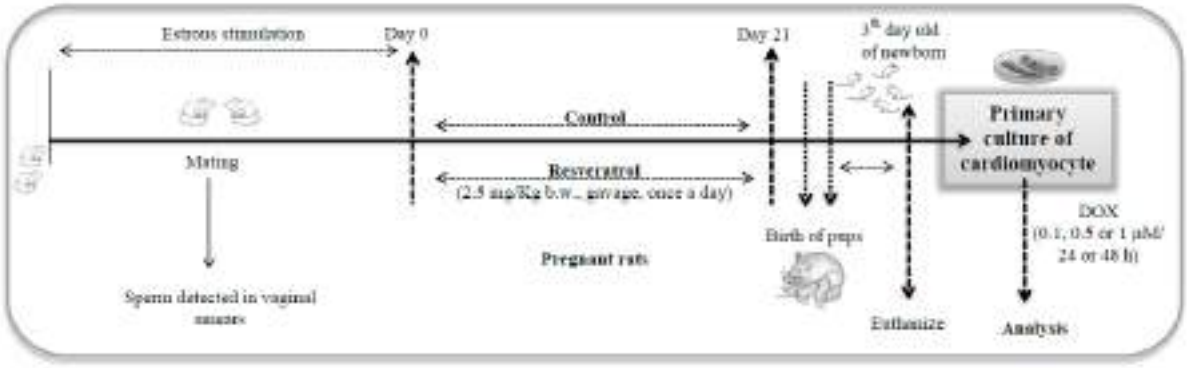


**Figure 1**: Experimental design and treatment schedule.

*2.5. Primary cardiomyocytes culture*

Primary culture was prepared from hearts of newborn rats with 3 days old and cultured as previously defined (Fu et al., 2005), with some modifications as follow. The hearts were removed, washed with PBS, minced and defragmented with a buffer containing 0.125% pancreatin and 0.3% BSA diluted in 10 mL (g/L): 80 NaCl, 2 KCl, 0.5 $Na_2HPO_4$, 10 $NaHCO_3$, and 20 dextrose (pH 7.2). This homogenate was digested at 37°C during repeated cycles of 5 min. The supernatant of which cycle was centrifuged (500 g, 5°C, 5 min) and pellet was resuspended in 3 mL DMEM containing 10% SFB and 1% penicilin/estreptomicin, and placed in a humidified incubator (5% $CO_2$, 37°C). This process was repeated until complete defragmentation of all cardiac tissue. At final, the pool of cells containing fibroblasts and cardiomyocytes were plating in 75 $cm^2$ bottle culture, for fibroblasts adhesion. After, the cellular suspension containing cardiomyocytes was aspired, centrifuged and finally plated in culture plates (Falcon, EUA) previously treated with gelatin (0.1% in PBS) for cardiomyocytes adhesion.

After the suitable confluence the culture was treated with DOX (0.1, 0.5 or 1.0 µM) during 24 or 48 h for the specific analysis.

*2.6. Trypan blue (TB) exclusion assay*

Cell viability was measured by TB exclusion test, as previously described (Robichová and Slameňová, 2002). In brief cardiomyocytes were treated 24 or 48 h with DOX (0.1, 0.5 or 1.0 µM), were washed with PBS, trypsinized, centrifuged and resuspended in PBS. An aliquot of this cellular suspension was stained with trypan blue dye (0.4%), and the number of viable (uncolored) and dead (colored) cells was counted in Automated Cell Counter (Countess®). The ratio of (viable cells/total cells) x100 results in the percentage of viable cells.

*2.7. Flow cytometric analysis*

Annexin V-PE was used together with a vital dye, 7-AAD, to distinguish apoptotic from necrotic cells, according to the manufacturer's instructions. Cardiomyocytes treated during 24 or 48 h with DOX (0.1, 0.5 or 1.0 µM) were washed with PBS, trypsinized, centrifuged and resuspended in 100 µL of binding buffer with 3 µL Annexin V-PE and 3 µL of 7-AAD, and incubated for 15 min in the dark and at room temperature. After incubation, 200 µL of binding buffer was added and mixed for immediate analysis. Data were collected and analyzed by a FACS Calibur flow cytometer with CellQuest software, with a maximum of 5000 events per sample. Fluorescence was measured and the percentage of viable, early apoptotic, late apoptotic and necrotic cells was determined.

*2.8. Comet assay*

The alkaline comet assay was performed as previously described (Hartmann and Speit, 1997; Singh et al., 1988) with minor modifications. Cardiomyocytes treated during 24 or 48 h with DOX (0.1, 0.5 or 1.0 μM) were washed with PBS, trypsinized, resuspended in complete medium, centrifuged and resuspended again in 200 μL PBS. Then, 30 μL of cell suspension was dissolved in 0.75% LMP agarose and spread onto a glass microscope slide pre-coated with a layer of 1% NMP agarose. The slides were then incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10.0) at 4 °C for at least 1 day in order to remove cellular proteins and membranes, leaving the DNA as 'nucleoids'.

In the modified comet assay, slides were removed from the lysis solution, washed in enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM $Na_2EDTA$, 0.2 mg/mL BSA, pH 8.0), and incubated with 100 μL FPG or EndoIII enzymes (300 mU per gel; 45 min 37°C). After, the slides were placed on a horizontal electrophoresis unit containing freshly made alkaline buffer (300 mM NaOH, 1 mM EDTA, pH 13.0), which covered the slides for 20 min at 4°C in order to allow unwinding of DNA and expression of alkali-labile sites. Subsequently, an electric current was applied for 20 min to allow DNA migration. Slides were then neutralized (0.4 M Tris, pH 7.5) and stained according to a silver-staining protocol (Nadin et al., 2001). After dry, one hundred cells were analyzed visually with an optical microscope, and scored according to tail length into five classes, assigned as class 0: undamaged, without a tail, to class 4: comets with no head, almost all DNA in tail. The genotoxic effect of DOX on cardiomyocytes was estimated by damage index (DI) of DNA, which ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) (Hartmann et al., 2003).

*2.9. Cardiomyocyte protein extracts*

After treatment of cardiomyocytes during 24 or 48 h with DOX (0.1, 0.5 or 1.0 µM) cells were washed with ice-cold PBS and whole cell protein extracts were obtained by scraping cells in RIPA buffer containing complete Mini protease cocktail inhibitor tablet (Roche Applied Science, QC, Canada). The protein extracts of cardiomyocytes were used for additional analysis.

*2.10. 2',7'-Dichlorofluorescein diacetate (DCF-DA) oxidation assay*

ROS production was assessed according previously described (LeBel et al., 1992) using DCF-DA as marker. In brief, 160 µL DCF-DA was incubated with 40 µL of cardiomyocyte protein extracts during 30 min at 37°C in dark. Fluorescence intensity was measured in SpectraMax M2e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California) using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (1 mM) and the levels of ROS were calculated as ηmol DCF formed/mg protein.

*2.11. Total sulfhydryl content*

This assay is based on the reduction of DTNB by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (ELLMAN, 1959). Sulfhydryl content is inversely correlated to oxidative damage to proteins. A standard curve was plotted for each measurement using GSH as a standard and the results were expressed as µmol/mg protein.

*2.12. Superoxide dismutase (SOD) activity*

SOD activity was evaluated by quantifying the inhibition of superoxide-dependent autoxidation of epinephrine, verifying the absorbance of samples at 480 nm (Misra and Fridovich, 1972). The inhibition of autoxidation of epinephrine occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of epinephrine autoxidation and the specific activity is reported as SOD Units/mg protein.

*2.13. Catalase (CAT) activity*

CAT activity was assayed as previously described (Aebi, 1984), based on the disappearance of $H_2O_2$ at 240 nm. One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT Units/mg protein.

*2.14. Immunoblotting analysis*

Immunoblotting analysis were performed as previously described (Towbin et al., 1979). For this, 25 μg of cardiomyocyte proteins was separated by electrophoresis on a 12% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad), which was subsequently blocked with 5% BSA for 2 h, washed in TTBS (Tris-buffered saline (TBS) pH7.5, plus 0.05% Tween-20) and then incubated overnight at 4°C in TTBS solution containing the antibodies: anti-sirt6, anti-actin, and anti-CAT, both diluted 1:500. After primary antibody incubation, membranes were washed with TTBS and incubated with horseradish-peroxidase conjugated secondary antibodies (anti-rabbit IgG, anti-mouse IgG, and anti-goat IgG, both diluted 1:3000, in TTBS solution). The blot was then developed using a chemiluminescence ECL kit (Thermo Scientific, São Paulo/Brazil).

Immunoblots were quantified by scanning the films with a DNR Bio-Imaging Systems scanner (MF ChemiBis2.0®) and determining optical densities with the ImageJ 1.48v software (Wayne Rasband, National Institutes of Health, USA).

*2.15. Protein quantification*

The protein concentration of the cardiomyocyte protein extracts was determined with method previously described (Lowry et al., 1951) using BSA as the standard.

*2.16. Statistical analysis*

The normal distribution of variables was tested with Kolmogorov-Smirnov normality test and the homogeneity of variances with Levene's test. Data were compared between groups by One-way Analysis of Variance (ANOVA) followed by Tukey post-hoc test. Correlations were performed by Pearson's correlation coefficient. The Statistical Package for the Social Sciences (SPSS, version 16.0) was used for all analyses, and the GraphPad Prism 5.03 program (GraphPad Software, San Diego, CA) was used as a computational tool for graph edition. Data were expressed as mean ± SD, and a p value of 0.05 was considered significant.

## 3. Results

*3.1. Resveratrol protects neonatal cardiomyocytes from DOX-induced death*

Viability of the neonatal cardiomyocytes, evaluated by TB exclusion test, is represented in **Figure 2**. It was observed a concentration-dependent cell death induced by DOX, both at 24 and 48 h after treatment. Maternal resveratrol supplementation significantly protected neonatal

cardiomyocytes from death induced with 0.5 and 1.0 µM DOX after 48 h (**Figure 2B**), but not after 24 h (**Figure 2A**), compared with neonatal cardiomyocytes from control mothers.

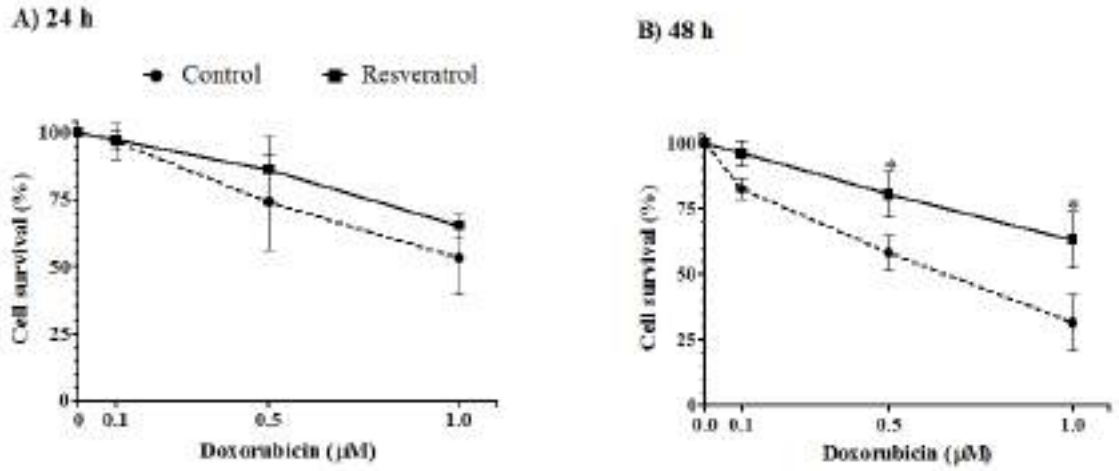


**Figure 2**: Effects of maternal resveratrol supplementation during pregnancy on neonatal cardiomyocytes viability. Cells were treated with DOX (0.1, 0.5 or 1.0 µM) for 24 h (A) or 48 h (B). Values are mean ± SD (n=8). * indicates $p<0.05$ from control group, by One-Way ANOVA, post-hoc Tukey.

*3.2. DOX-induced apoptosis and necrosis are reduced in neonatal cardiomyocytes from resveratrol supplemented rats during pregnancy*

In an attempt to understand the main mechanism of DOX-induced death in this model, and the effects of resveratrol supplementation, neonatal cardiomyocytes treated with DOX were analyzed by flow cytometry (**Figure 3**). Likewise to the viability results, it was observed a concentration-dependent increase in cardiomyocytes death (24 and 48 h after DOX treatment), and resveratrol did not protect against DOX-induced cell death 24 h after treatment (**Figure 3 A-D**). Conversely, the neonatal cardiomyocytes from resveratrol supplemented pregnant rats exhibited a significant protection against DOX-induced death 48 h after treatment with all concentrations of DOX, with an increase in total viable cells and decrease of apoptotic and necrotic cells (**Figure 3 E-H**). These results demonstrate that apoptosis is the main mechanism of

DOX-induced death in neonatal cardiomyocytes, and that this effect is significantly reduced by the resveratrol supplementation during pregnancy.

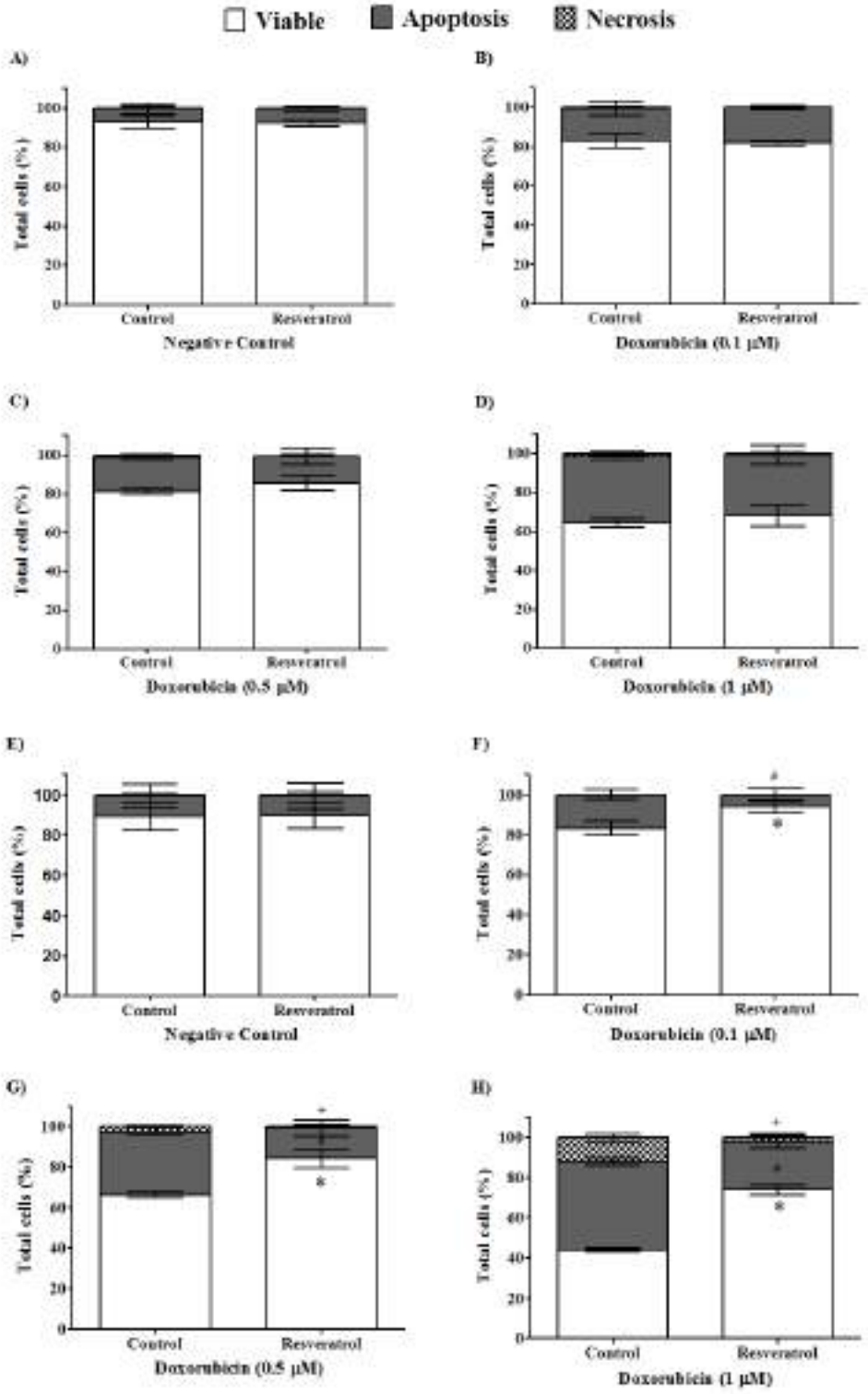

**Figure 3**: Effects of maternal resveratrol supplementation during pregnancy on neonatal cardiomyocytes viability, apoptosis, and necrosis by flow cytometry analysis. Cells were treated 24 h (A-D) or 48 h (E-H) with DOX (0.1, 0.5 or 1.0 µM). Negative control = cells without DOX. Values are mean ± SD (n=8). Symbol * indicates viable, # apoptotic and + necrotic cells, with $p<0.05$ from control group, by One-Way ANOVA, post-hoc Tukey.

*3.3. Resveratrol protects neonatal cardiomyocytes against increase in ROS production*

As can be seen in **Figure 4**, neonatal cardiomyocytes exposed to DOX (0.5 and 1.0 µM) showed a significant increase in ROS production in relation to cells not exposed to the drug (negative control). Maternal resveratrol supplementation during pregnancy did not protect neonatal cardiomyocytes 24 h after DOX treatment. A significant increase in ROS production induced by DOX was also observed 48 h after treatment of neonatal cardiomyocytes from control group with 0.5 and 1.0 µM DOX (**Figure 4B**). Moreover, resveratrol significantly protected these cardiomyocytes against the ROS increase induced by all DOX concentrations. Through Pearson's analysis, it was observed an inverse correlation between cell viability and ROS production in neonatal cardiomyocytes both at 24 and 48 h ($r = -0.8$, $p<0.0001$ and $r = -0.789$, $p<0.001$), respectively. Importantly, there was a significant direct correlation between ROS production and apoptosis ($r = 0.836$, $p<0.0001$, $r = 0.817$, $p<0.0001$) and necrosis ($r = 0.425$, $p<0.055$; $r = 0.637$, $p<0.001$) observed both at 24 and 48 h after DOX treatment, respectively.

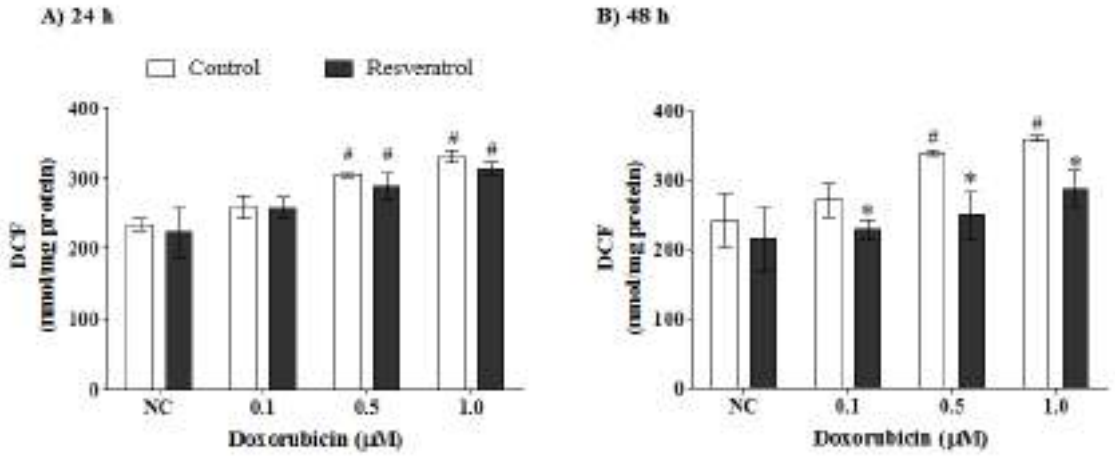

**Figure 4**: Effects of maternal resveratrol supplementation during pregnancy on ROS production in neonatal cardiomyocytes. Cells were treated with DOX (0.1, 0.5 or 1.0 µM) for 24 h (A) or 48 h (B). Values are mean ± SD (n=8). * indicates $p<0.05$ from control group and # indicates $p<0.05$ from negative control (NC – cells without DOX), by One-Way ANOVA, post-hoc Tukey.

### *3.4. Resveratrol supplementation during pregnancy induces a protective response against oxidative DOX-induced DNA damage*

Subsequently, the effects of DOX on DNA integrity of neonatal cardiomyocytes were evaluated by DNA damage analysis by alkaline Comet assay. This assay detects primary (repairable) DNA single and double-strand breaks and alkali-labile sites (Collins et al., 1993; Dušinská and Collins, 1996). The results show a concentration-dependent increase of DNA damage DOX-induced, observed in neonatal cardiomyocytes from all groups of pregnant rats (**Figure 5 A-B**). However, the maternal supplementation with resveratrol induced a significant protection of neonatal cardiomyocytes, both at 24 and 48 h after treatment with DOX (except 1.0 µM for resveratrol supplemented mothers). Moreover, it was observed a positive effect of resveratrol on cardiomyocytes DNA damage even in cells not treated with DOX (negative control).

Subsequently we performed analysis with the DNA-repair enzymes EndoIII and FPG, which increase the Comet test specificity and are able to recognize oxidative base damaged and convert it into single-strand breaks (Collins et al., 1993; Hartmann et al., 2003). The results show the magnitude of oxidative DNA damage caused by DOX treatment in neonatal cardiomyocytes (**Figure 5 C-F**). The oxidative damage was recognized by EndoIII and FPG and was significantly lower in neonatal cells from supplemented mothers in relation to neonatal cells from controls. Moreover, at same form observed in alkaline Comet assay, resveratrol supplementation was able to decrease oxidative DNA damage in neonatal cardiomyocytes not exposed to DOX, both after 24 and 48 h after DOX treatment. Importantly, Pearson's analysis demonstrates a direct

correlation between EndoIII or FPG activity, and ROS production in neonatal cardiomyocytes both at 24 h ($r = 0.804$, $p<0.0001$ and $r = 0.754$, $p<0.0001$) and 48h ($r = 0.798$, $p<0.0001$ and $r = 0.790$, $p<0.0001$) after DOX treatment, for EndoIII and FPG, respectively.

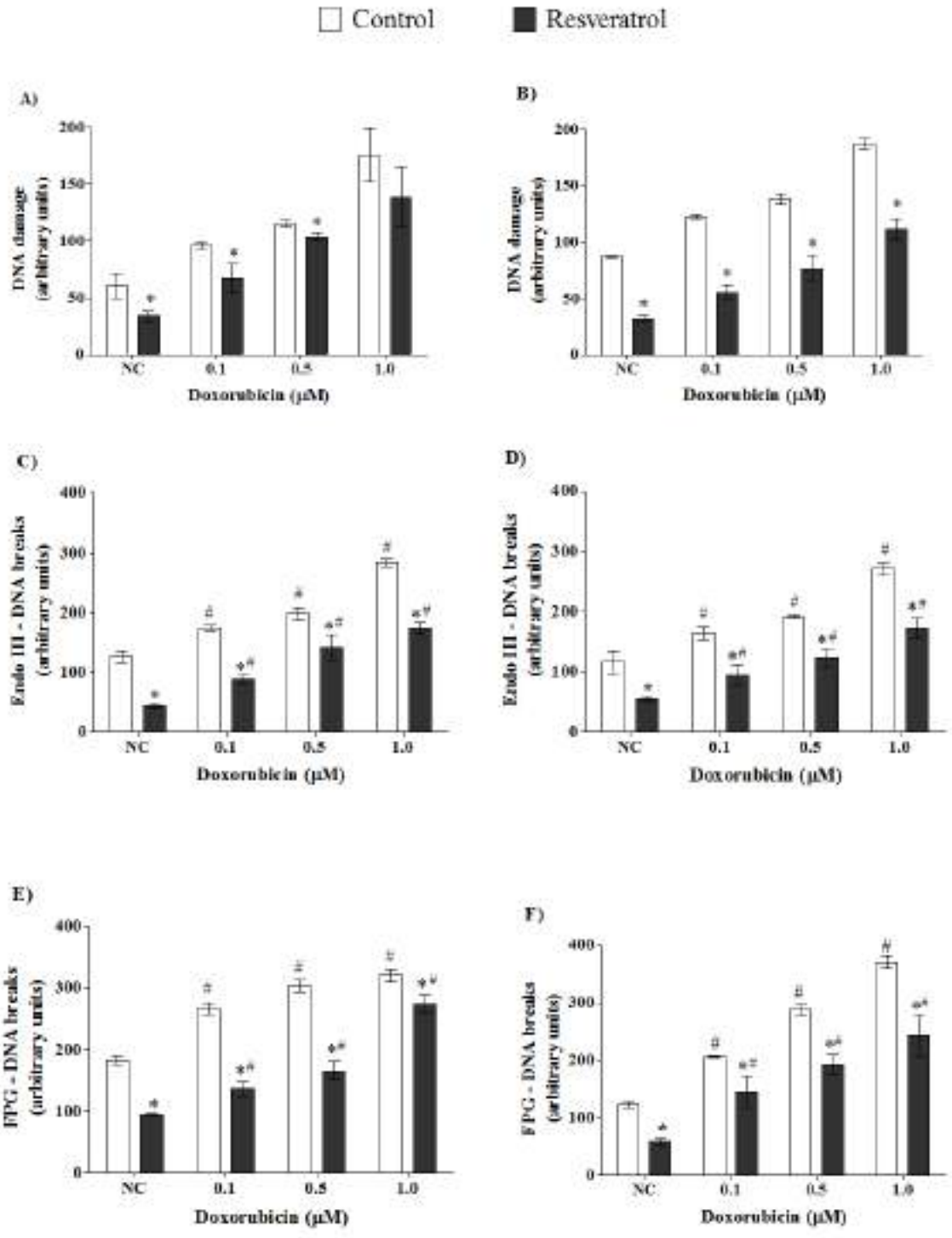

**Figure 5**: Effects of maternal resveratrol supplementation during pregnancy on DNA damage of neonatal cardiomyocytes. Cells were treated with DOX (0.1, 0.5 or 1.0 μM) for 24 h (A) or 48 h (B) and DNA damage due to single and double-strand breaks and alkali-labile sites were detected. Effects of resveratrol on oxidative DNA damage were analyzed with EndoIII and FPG enzymes when neonatal cardiomyocytes were treated with DOX (0.1, 0.5 or 1.0 μM) for 24 h (C and E) or 48 h (D and F). These enzymes recognize oxidative base damaged and convert it into single-strand breaks. Values are mean ± SD (n=8). * indicates $p<0.05$ from control group, and # indicates $p<0.05$ from negative control (NC - cells without DOX), by One-Way ANOVA, post-hoc Tukey.

*3.5. Resveratrol protects neonatal cardiomyocytes of the decrease in antioxidant defenses and thiol content induced by DOX*

To examine whether the increase in DOX-induced ROS generation is associated with decreased activities of antioxidant enzymes and total thiol content, we investigated the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) (**Table 1**), as well as total sulfhydryl content (**Figure 6**). These results demonstrated that treatment with DOX induced a significant decrease of SOD activity in neonatal cardiomyocytes from control group compared to the resveratrol supplemented rats, both at 24 and 48 h after DOX treatment. At same form, DOX induced a significant decrease in CAT activity levels, which was concentration-dependent, and more pronounced in neonatal cardiomyocytes from control group (**Table 1**). Notably, resveratrol protected neonatal cardiomyocytes from decrease in total sulfhydryl content DOX-induced in relation to cells from control group, both at 24 and 48 h after DOX treatment (**Figure 6**). However, the decrease in sulfhydryl content was more pronounced at higher concentrations, without protective effect of resveratrol at 1.0 μM DOX.

Moreover, the activity of antioxidant enzymes was significantly correlated whit cell viability, as well as ROS production. Particularly, CAT activity showed an inverse correlation with ROS production ($r = -0.763$, $p<0.0001$ and $r = -0.808$, $p<0.0001$) and a direct correlation with cell viability ($r = 0.508$, $p<0.007$ and $r = 0.680$, $p<0.001$) both at 24 and 48 h after DOX

treatment, respectively. This effect was also observed for SOD activity, which correlates inversely with ROS production both at 24 and 48h after DOX treatment ($r = -0.527$, $p<0.004$ and $r = -0.671$, $p<0.0001$), respectively. In addition, there was a significant direct correlation between SOD activity and cell viability ($r = 0.398$, $p = 0.024$; $r = 0.558$, $p<0.001$) both at 24 and 48h after DOX treatment.

**Table 1:** Effects of resveratrol supplementation during pregnancy on SOD and CAT activities of neonatal cardiomyocytes.

| | ***Control*** | ***Resveratrol*** | ***Control*** | ***Resveratrol*** |
|---|---|---|---|---|
| | ***SOD (U/mg protein)*** | | | |
| ***DOX (µM) treatment*** | ***24 hours*** | | ***48 hours*** | |
| **NC** | 4.61 ± 0.54 | 6.26 ± 1.36* | 4.46 ± 0.30 | 6.00 ± 1.63 |
| **0.1** | 3.90 ± 0.68 | 5.31 ± 0.38* | 3.98 ± 0.08$^{\#}$ | 6.06 ± 1.13* |
| **0.5** | 3.22 ± 0.72$^{\#}$ | 5.22 ± 0.84* | 3.03 ± 0.74$^{\#}$ | 5.12 ± 0.95* |
| **1.0** | 2.83 ± 0.40$^{\#}$ | 5.56 ± 0.72* | 2.53 ± 0.93$^{\#}$ | 5.19 ± 1.39* |
| | ***CAT (U/mg protein)*** | | | |
| ***DOX (µM) treatment*** | ***24 hours*** | | ***48 hours*** | |
| **NC** | 12.15 ± 2.16 | 24.07 ± 2.93* | 12.30 ± 0.93 | 27.11 ± 2.55* |
| **0.1** | 6.16 ± 0.81$^{\#}$ | 13.00 ± 4.29*$^{\#}$ | 7.85 ± 1.03$^{\#}$ | 13.56 ± 2.62*$^{\#}$ |
| **0.5** | 4.04 ± 0.57$^{\#}$ | 9.47 ± 2.53*$^{\#}$ | 5.35 ± 0.62$^{\#}$ | 12.24 ± 3.87*$^{\#}$ |
| **1.0** | 2.62 ± 0.20$^{\#}$ | 8.78 ± 4.15*$^{\#}$ | 2.68 ± 1.51$^{\#}$ | 8.36 ± 1.79*$^{\#}$ |

Cells were treated with DOX (0.1, 0.5 or 1.0 µM) during 24 or 48 h. Values are mean ± SD (n=8). * indicates $p<0.05$ from control group, and # indicates $p<0.05$ from negative control (NC - cells without DOX), by One-Way ANOVA, post-hoc Tukey.

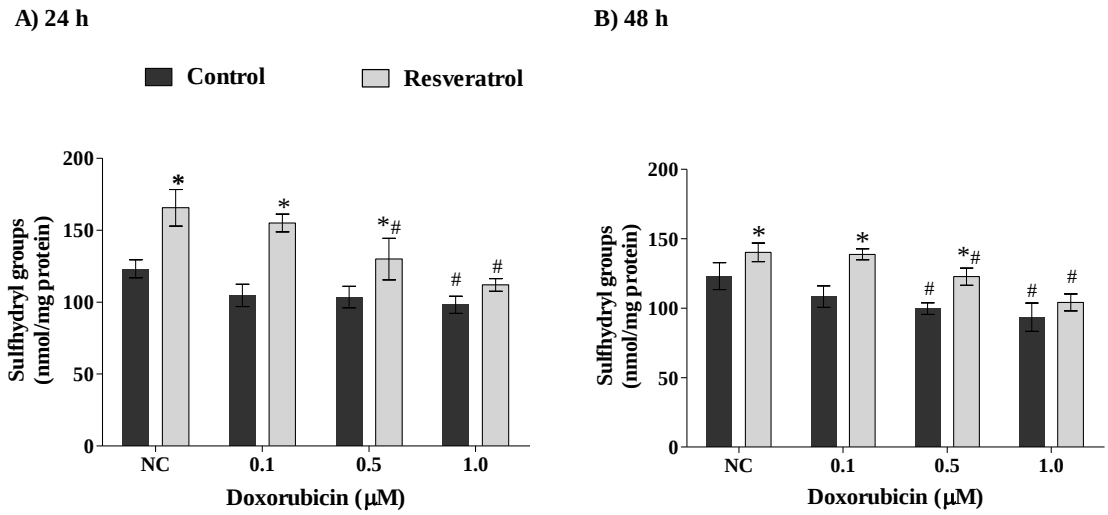


**Figure 6**: Effects of maternal resveratrol supplementation during pregnancy on total sulfhydryl groups of neonatal cardiomyocytes. Cells were treated with DOX (0.1, 0.5 or 1.0 μM) for 24 h (A) or 48 h (B). Values are mean ± SD (n=8). * indicates $p<0.05$ from control group and # indicates $p<0.05$ from negative control (NC – cells without DOX), by One-Way ANOVA, post-hoc Tukey.

### *3.6.Levels of Sirt6 and CAT expression are increased in neonatal cardiomyocytes from resveratrol supplemented rats during pregnancy*

Immunoblotting analysis showed that resveratrol supplementation of rats during pregnancy induced a significant increase in expression of Sirt6 protein of neonatal cardiomyocytes in relation to neonatal cells from controls (**Figure 7A**, **B** and **C**). However, the increase in Sirt6 expression was only observed in neonatal cells exposed to DOX, with an increase dependent of DOX concentration (**Figure 7A** and **C**). On the other hand, the effect of resveratrol on CAT expression was slight and a significant difference between groups was only observed in neonatal cardiomyocytes exposed to low concentration or not exposed to DOX (**Fig 7B and C**). At high DOX concentrations the effect of resveratrol on CAT expression was similar to that observed in neonatal cardiomyocytes from control group.

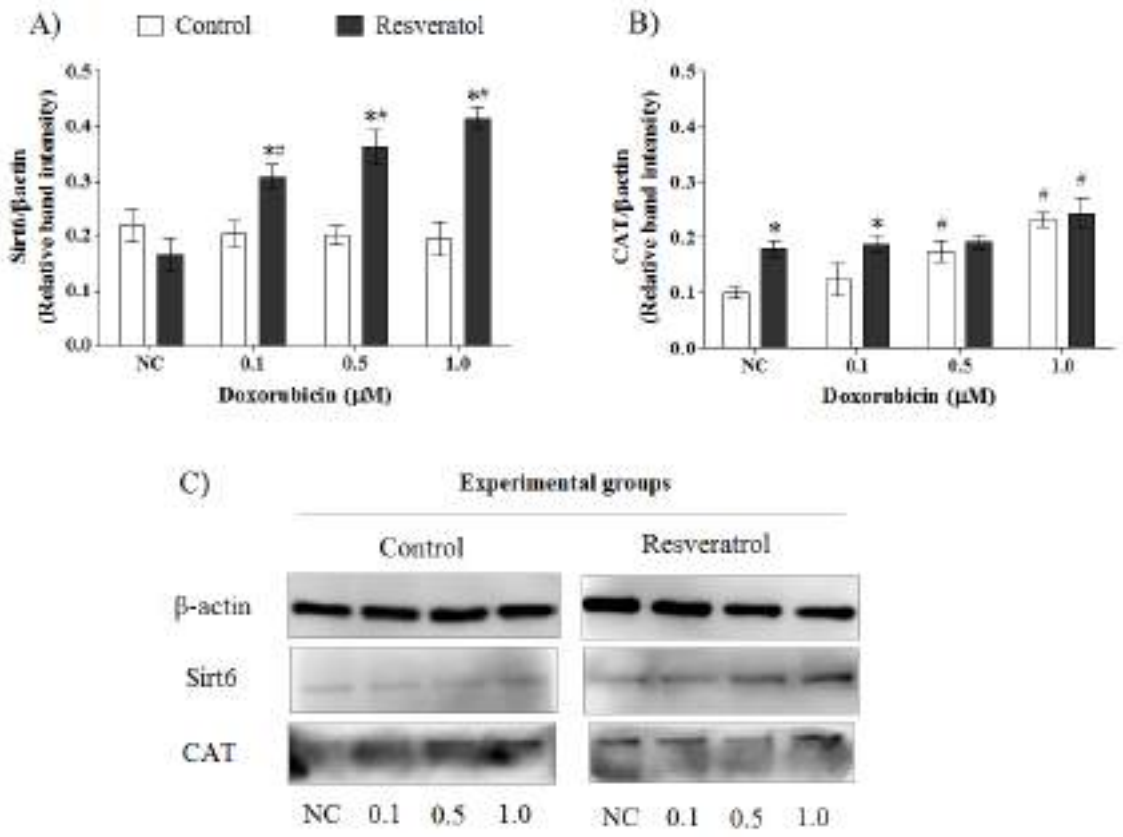


**Figure 7**: Effects of maternal resveratrol supplementation during pregnancy on Sirt6 and CAT protein expression of neonatal cardiomyocytes. Cells were treated with DOX (0.1, 0.5 or 1.0 μM) for 48 h. Bar graph corresponds to mean ± SD of the quantification values of Sirt1/β-actin ratio (A) and CAT/β-actin ratio (B) from all samples. * Indicates $p<0.05$ from control group, and # indicates $p<0.05$ from negative control (NC – cells without DOX), by One-Way ANOVA, post-hoc Tukey.

## 4. Discussion

DOX is widely used to treat childhood and adult cancers, but its chemotherapeutic dose is limited by both acute and chronic cardiotoxicity, with cumulative cardiotoxic effects (Aleman et al., 2007; Bristow et al., 1981; Grenier and Lipshultz, 1998). Approximately 60% of pediatric cancer patients are treated with anthracyclines and about 10% of them develop symptoms of cardiomyopathy up to 15 years after the end of chemotherapy (Kremer et al., 2002). The development of novel therapeutic strategies to reduce the treatment outcomes is essential,

viewing the increase of life expectancy for decades after the anti-cancer therapy. Studies in the field of experimental and clinical research, as well as preventive medicine have highlighted the beneficial effects of resveratrol on cardiovascular and metabolic diseases (Cai et al., 2015; Catalgol et al., 2012; Das and Maulik, 2006). Moreover, resveratrol is a bioactive component of diet that, among other environmental stimuli, can modulate epigenetic factors. In this study, for to determine the possible cardioprotective heritable effects of resveratrol against DOX-induced toxicity, we evaluated at first time the cardioprotective effects of maternal resveratrol supplementation during pregnancy on DOX-induced cardiotoxicity in neonatal heart.

Neonatal cardiomyocytes from control pregnant rats exhibited a pronounced DOX-induced concentration-related cardiotoxicity, with significant decrease in cell viability, increase in apoptotic and necrotic cell death, which were strongly correlated with the increase in ROS production and decrease in activity of antioxidant defense systems. Apoptosis was the main mechanism of DOX-induced cardiomyocytes death that is consistent with the elucidated mechanisms of DOX toxicity. Neonatal cardiomyocytes from controls also showed lower levels of total sulfhydryl groups and a significant increase in oxidative DNA damage DOX-induced. However, the maternal supplementation with 2.5 mg/Kg resveratrol per day, during the gestational period was effective in protecting the neonatal heart against DOX-induced cardiotoxicity. These results are favorable to the hypothesis that maternal supplementation with resveratrol during pregnancy can modulate the responses to stressful agents of progeny.

The global demand for more reasonable therapeutics has identified in resveratrol important features to human health, and it still possesses an effective cost, exhibits low toxicity, and is readily available (Aggarwal et al., 2011; Li and Vederas, 2009). This bioactive compound is widely known by its protective effect against oxidative stress in different tissues and in pathological conditions such as cardiovascular diseases, inflammatory response, cancer and

diabetes (Boocock et al., 2007; Das and Das, 2007; Movahed et al., 2012; Silan, 2008; Tao et al., 2016), particularly in diseases where oxidative stress plays an important role. However, the interest in this compound has been renewed in recent years, first from its identification as a chemopreventive agent for skin cancer, regulating multiple cancer-inflammation pathways (Aggarwal et al., 2011; Amiri et al., 2013; Aziz et al., 2005), and subsequently from reports where it can activate epigenetic cofactors, particularly sirtuin deacetylases and extends the lifespan of some animal species (Chung et al., 2011; Howitz et al., 2003; Lagouge et al., 2006; Zhang et al., 2011).

In this research, resveratrol supplementation of pregnant rats induced a protective effect on neonatal cardiomyocytes exposed to DOX, with an increase in cell viability, decrease in apoptotic and necrotic cell death, which were correlated with the decrease in ROS production both at 24 and 48 hours. Moreover, maternal supplementation with resveratrol prevented in neonatal cardiomyocytes the decrease of SOD activity DOX-induced, as well as lead to an increase in CAT activity, with a slight upregulation of CAT protein expression. Besides antioxidant enzymes, the total sulfhydryl content- a molecular target of ROS in the non-enzymatic antioxidant system- was also altered in neonatal cardiomyocytes from resveratrol supplemented rats during pregnancy, in which the resveratrol increases the total sulfhydryl content and counteracts the oxidative effects induced by DOX at lower concentrations (NC, 0.1 and 0.5 μM).

Resveratrol is a stilbenoid compound that consists of two aromatic rings, which are attached by a methylene bridge. It is present in *cis/trans* isoforms both of which may be glycosylated and the *trans* isomer has the major biological activity (Ovesná and Horváthová-Kozics, 2005). Due to the aromatic groups in its structure, resveratrol is able to function as antioxidant and prevent oxidation reactions. Resveratrol is able to sequester 2,2-azinobis(3-

ethylbenzthiazoline-6-sulfonic acid; ABTS) and 1,1- diphenyl-2-picrylhydrazyl (DPPH), scavenging hydroxyl radicals (Soares et al., 2003). In addition, in vascular system, this molecule can also scavenge $H_2O_2$, delaying the oxidative stress and preventing endothelial cell death induced by ROS, which are responsible, at least in part, for its cardioprotective effects (Ungvari et al., 2007).

Since resveratrol treatment has been shown to be safe in pregnancy and it is able to cross the placental barrier, affecting the fetus directly (Bourque et al., 2012; Singh et al., 2011; Williams et al., 2009), it is possible that the cardioprotective effects observed in this study might be a direct scavenging action of resveratrol on DOX-induced ROS production. Moreover, the decrease in ROS production can also be due to an upregulation of enzymatic and non-enzymatic antioxidant defense system, which in turn counteracts the futile cycle of ROS production during DOX mitochondrial metabolization.

Several mechanisms have been proposed for DOX-mediated cell death in clinically relevant doses. DOX can act as a DNA intercalator, forming DOX-DNA adducts, which can activate DNA damage responses and induce cell death independent of topoisomerase II (Forrest et al., 2012; Swift et al., 2006); or can act as a topoisomerase II poisoning, resulting in double-strand DNA breaks and cell death (Pommier et al., 2010); DOX can mediate the cell death through oxidative stress production, once DOX-induced release of free radicals may cause oxidative stress, resulting in DNA damage and cell death (Berlin and Haseltine, 1981; Minotti et al., 2004; Šimůnek et al., 2009); or still through ceramide overproduction, which is a lipid molecule involved in a variety of cellular processes including growth arrest, apoptosis, and senescence (Senchenkov et al., 2001). In many of these proposed mechanisms overproduction of the oxidative stress and DNA damage has been present.

Neonatal cardiomyocytes treated with DOX exhibited a concentration-related increase in oxidative DNA damage, which is related to the oxidative stress, measured through the activities of EndoIII and FPG enzymes. Both act as glycosilase enzymes, while EndoIII recognizes oxidized pyrimidines in DNA, the FPG has the ability to recognize imidazole-ring-opened purines, or formamidopyrimidines (fapy Ade and fapy Gua), which occur during the spontaneous breakdown of damaged purines (Boiteux, 1993; Doetsch et al., 1987; Dušinská and Collins, 1996). Our research demonstrated that resveratrol supplied a notable cardioprotection against DOX-induced DNA damage, preserving the DNA integrity. Neonatal cardiomyocytes from resveratrol supplemented mothers, both after 24 and 48 h of DOX treatment, showed a significant decrease in oxidative DNA strand-breaks, following EndoIII or FPG incubation, which represents a decrease in residual oxidized DNA bases. These data were supported by Pearson's correlation analysis, which demonstrated a direct correlation between EndoIII or FPG activities and DOX-induced ROS production.

In the analysis of DOX-induced DNA damage, it is important to note that the decrease in the oxidative DNA strand-breaks in neonatal cardiomyocytes from resveratrol supplemented mothers was also observed in the absence of DOX treatment. This can be a result of a direct antioxidant action of resveratrol, once it is able to cross to placental barrier. However, we wondered whether the maternal supplementation with resveratrol during pregnancy could activate, via modulation of epigenetic factors, DNA damage response pathways in neonatal cardiomyocytes. Among the most studied epigenetic factors, it has been demonstrated that resveratrol can modulate the activity of Sirt1, increasing lifespan in yeast, worms, and flies and enhancing health span in rodents (Baur et al., 2006; Howitz et al., 2003; Wood et al., 2004). Recently, Sirt6 is another histone deacetylase that has received attention by its key role in the

DNA repair and maintenance of genomic stability (Kaidi et al., 2010; Mao et al., 2011; Mostoslavsky et al., 2006; Toiber et al., 2013).

Our results demonstrated that maternal supplementation with resveratrol induced a significant increase in Sirt6 expression of neonatal cardiomyocytes exposed to DOX, which was concentration-related. The expression of Sirt6 was not altered in neonatal cardiomyocytes from control pregnant rats. Moreover, resveratrol did not alter the Sirt6 expression in neonatal cells not exposed to DOX. Concerning the cardioprotective action of resveratrol on DOX-induced DNA damage in neonatal cells, these results can be due, at least partially, to Sirt6 overexpression, which in turn acts as a scaffold protein in DOX-induced DNA damage repair. Sirt6 is a chromatin-bound protein with important function on genome stability that following DNA damage is rapidly recruited to sites of double strand breaks, activating downstream DNA damage repair factors and the efficient repair through both non-homologous end-joining and homologous recombination (Cai et al., 2012; Mao et al., 2011; Toiber et al., 2013). Moreover, Sirt6 binds to, and mono-ADP-ribosylates PARP-1, a member enzyme of PARP (poly-ADP-ribose polymerase) family, which plays an important role in regulation of various cellular and subcellular processes, including DNA repair, gene expression, genomic stability, cell cycle, and cell death (Mao et al., 2011; Schreiber et al., 2006).

Similarly to other HDACs class III, Sirt6 is dependent on $NAD^+$, a classical coenzyme with well-established role in cellular redox reactions. However, $NAD^+$ can be consumed by many families of enzymes, including PARPs, when the PARP-1 is the major $NAD^+$ consuming enzyme (Schreiber et al., 2006). Moreover, it has been demonstrated that $NAD^+$ levels decreases and Sirt6 is inactivated during cardiac hypertrophy, indicating a relationship between $NAD^+$ and Sirt6 in heart (Cai et al., 2012). The cardioprotection awarded by resveratrol in this model can be due to the increase in $NAD^+$ levels. Resveratrol inhibits mitochondrial ATP synthase activity by binding

to its G-subunit and interfering with mitochondrial respiration (Gledhill et al., 2007; Zheng and Ramirez, 2000). As consequence, resveratrol causes an increase in the ratio of AMP to ATP (AMP/ATP), and then activates the energy-sensing AMPK (AMP-activated protein kinase) (Dolinsky et al., 2009; Hart et al., 2013), which in turn increases $NAD^+$ that may serve as a metabolic sensor leading to Sirt6 activation. Particularly, it was also shown that resveratrol protects mouse embryonic fibroblasts against DOX-induced cardiotoxicity through activation of AMPK (Wang et al., 2012), which is related to decrease in ROS production and cardioprotective effect against glucotoxicity in adult cardiomyocytes (Balteau et al., 2014).

On the other hand, Sirt6 expression was not altered in neonatal cardiomyocytes from control pregnant rats, which also exhibited an increase in DOX-induced oxidative DNA-damage and ROS production, suggesting that the lack of cardioprotection is dependent of Sirt6 expression. It has been demonstrated that during oxidative stress situations PARP-1 is activated and competes with Sirt6 for a limited pool of $NAD^+$ in the cell. In view of the very low $K_m$ and high $V_{max}$ of PARP-1 (Mendoza-Alvarez and Alvarez-Gonzalez, 1993), few $NAD^+$ is available for the Sirt6, limiting its activity. However, surprisingly maternal resveratrol supplementation did not alter Sirt6 expression in neonatal cardiomyocytes not exposed to DOX. A possible explanation for this could be that Sirt6 is a defense protein and cells try to activate defense pathways to survive, which occurs under stressful events such as hypoxic damage to heart (Maksin-Matveev et al., 2015), cardiac hypertrophy (Cai et al., 2012), premature chondrocytes senescence (Nagai et al., 2015), and here DOX-induced toxicity to neonatal heart.

Therefore, in this research we investigated the cardioprotective effects of resveratrol on DOX-toxicity with a novel approach, and we demonstrated, for the first time, that maternal supplementation during pregnancy is able to protect neonatal cardiomyocytes against DOX-toxicity. Some of the beneficial cardiovascular effects of resveratrol are mediated by the

overexpression of Sirt6 and increase in DNA damage response. These effects together with the modulation of antioxidant enzymes and reduction in cellular oxidative stress, contribute to the cardiomyocytes survival under DOX toxicity. A representative scheme of cardioprotection in this model is presented in **Figure 8**.

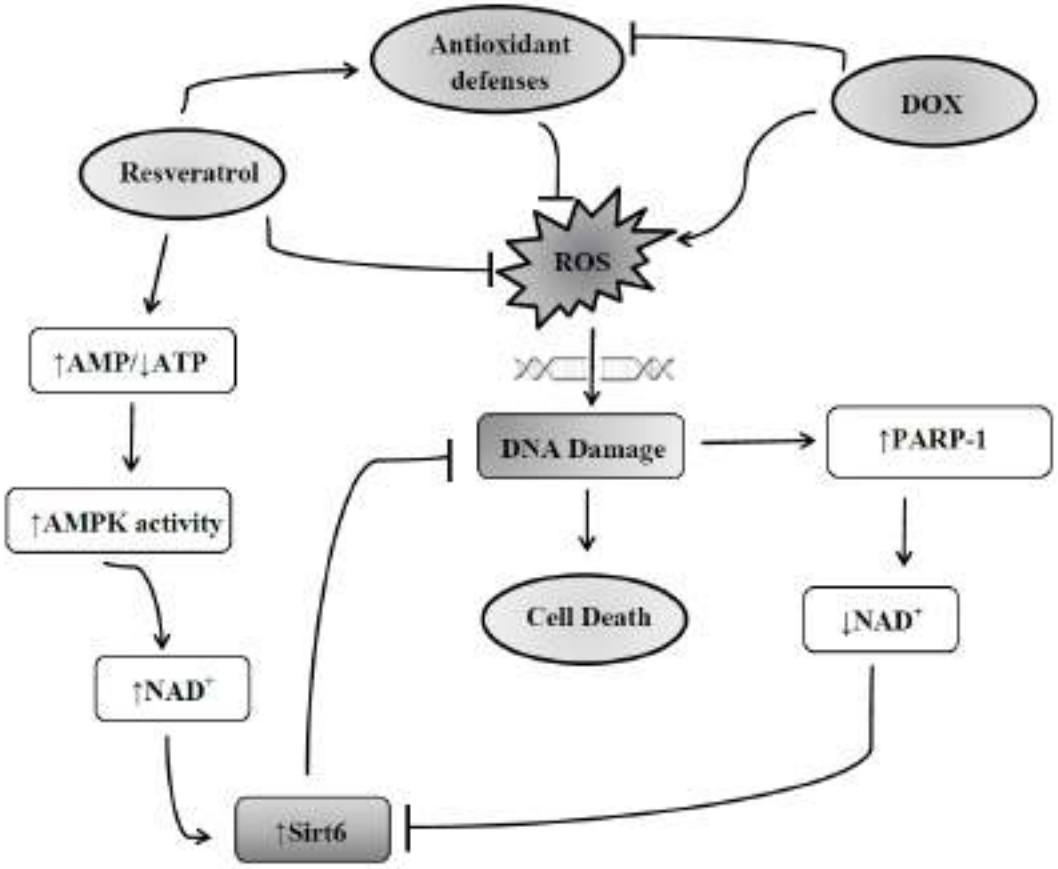


**Figure 8**: The suggested mechanism for the cardioprotective effects of maternal resveratrol supplementation on neonatal cardiomyocytes exposed to DOX. Resveratrol can protect the neonatal cardiomyocytes of DOX-induced DNA damage blocking the ROS production by an increase in antioxidant defense, or acting as a scavenger of ROS. Moreover, resveratrol or its metabolites can act on mitochondria reducing ATP synthesis. As a result, the ratio of AMP to ATP increases. The increase in AMP activates AMP kinase, which in turn increases $NAD^+$ levels, which activates the Sirt6. This then leads to the various effects of Sirt6, such as reduction in the DNA damage and cardiomyocytes death. On

the other hand, the DOX-induced DNA damage can increase the activity of PARP-1, which competes with Sirt6 for $NAD^+$ storage.

Additional studies are necessary to determine the role of Sirt6 desacetilation targets and PARP-1 in this resveratrol cardioprotective model against DOX toxicity. In summary, our findings demonstrate that neonatal cardiomyocytes from maternal supplemented rats during pregnancy are notably more resistant to DOX-induced toxicity, which occurs by means of regulation of oxidative stress through antioxidant defense system, and genome stability by Sirt6 overexpression, denoting an important involvement of maternal environment in answers to stressful agents of progenies throughout life.

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**Conflict of interest**

The authors have declared no conflicts of interest.

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# 6. DISCUSSÃO

Mostramos nesse trabalho, em modelo experimental animal, que o exercício físico ou a suplementação com resveratrol realizados pela genitora durante o período gestacional exercem efeito protetor no coração da prole contra a toxicidade induzida pelo quimioterápico DOX. Os resultados foram semelhantes para exercício e resveratrol, onde ambos protegeram contra a cardiotoxicidade gerada pela DOX nos cardiomiócitos dos neonatos. Os efeitos sobre a redução da morte celular por apoptose e necrose, aumentando a viabilidade dos cardiomiócitos, redução dos níveis de ERO e de dano oxidativo ao DNA, bem como aumento da atividade de enzimas antioxidantes SOD e CAT foram semelhantes entre as diferentes intervenções.

Ao explorar os mecanismos envolvidos nessa cardioproteção, podemos dizer que passa pela ação efetiva do sistema de defesa antioxidante, que resulta numa redução de ERO e do dano oxidativo ao DNA dos cardiomiócitos, mas também por uma maior sinalização para o reparo de dano ao DNA, com aumento na expressão da enzima Sirt6, principalmente em cardiomiócitos de neonatos de genitoras que realizaram exercício durante a gestação. Vale ressaltar que o aumento na atividade de defesas antioxidantes e redução de danos oxidativos são desfechos clássicos para o exercício e resveratrol, que justificam cardioproteção, mas, sobretudo quando essas intervenções são realizadas no próprio organismo onde a toxicidade será induzida.

No nosso modelo, as diferentes intervenções realizadas na mãe durante a gestação obtiveram resultados cardioprotetores que foram observados no coração do neonato da geração subsequente, e obtidos durante o desenvolvimento embrionário até o nascimento. Assim, além dos resultados clássicos de proteção pelo exercício e resveratrol contra agentes indutores de estresse oxidativo, é importante pensar que o mecanismo envolvido na proteção conferida aos neonatos expostos à DOX passe pela formação de uma herança materna de cardioproteção, transmitida de mãe para prole, e sobre esse tema também nos debruçamos na discussão dos resultados.

Durante muitos séculos, o homem deteve um conhecimento apenas empírico das fascinantes similaridades entre pais e filhos; conhecimento obtido através das

observações de filósofos gregos como Anaxágoras e Hipócrates (MAYR, 1985). E foi apenas no século XIX, com o surgimento das leis de Mendel, que o DNA foi identificado e sua sequência reconhecida como agente de hereditariedade (AVERY; MACLEOD; MCCARTY, 1944; HERSHEY; CHASE, 1952).

A associação entre genótipo e fenótipo, que constitui a essência da genética Mendeliana, procurou estimar a que ponto as diferenças genéticas individuais contribuíam para as variações individuais de fenótipo. Como as descobertas de Mendel tinham foco no DNA e sua sequência como centro da hereditariedade, outras formas de herança foram ignoradas durante muito tempo, e a nossa visão de hereditariedade estreitada invariavelmente às associações entre genótipo e fenótipo (MAYR, 1985).

Aristóteles, Lamarck, Darwin e muitos outros pensaram que uma via de transmissão do fenótipo de uma geração para outra poderia envolver a “herança de características adquiridas com exposições ambientais e experiências de vida” (MAYR, 1985). Essa proposta caiu por terra com as descobertas e leis de Mendel. Entretanto, a ideia da hereditariedade dos efeitos de exposições ambientais sobre as características fenotípicas e o risco para desenvolvimento de doenças nos descendentes fascinou os cientistas por muitas décadas. Desde então, diversos estudos mostraram que não apenas a alteração no material genético, mas também a influência de exposições ambientais podem modular fatores epigenéticos os quais conduzirão a alterações fenotípicas hereditárias transmitidas de mãe para filho, e que podem persistir por inúmeras gerações (NELSON; NADEAU, 2010; WHITELAW; WHITELAW, 2008; YOUNGSON; WHITELAW, 2008). Portanto, além da herança Mendeliana, duas outras formas de transmissão de informações hereditárias, baseadas em modificações epigenéticas e responsáveis pelo fenótipo, são importantes nesse contexto:

1. A herança epigenética intergeracional ou parental: resultante da exposição intrauterina ou de células germinativas a fatores ambientais com capacidade de modificar o epigenoma, como os fatores nutricionais, hormonais, o estresse, toxinas ambientais; sendo essa informação transmitida de uma geração à subsequente (DAXINGER; WHITELAW, 2012; HEARD; MARTIENSSEN, 2014);

2. A herança epigenética transgeracional: alteração fenotípica decorrente de uma modificação epigenética que persiste através de gerações em indivíduos que não foram diretamente expostos ao fator ambiental, ou que não herdaram a variação genética individual (DAXINGER; WHITELAW, 2012; HEARD; MARTIENSSEN, 2014).

No caso do nosso modelo, as ratas foram expostas ao exercício ou suplementação com resveratrol durante a gestação, e o feto pode ser afetado ainda no útero (1ª geração), ou as células germinativas do feto (que dariam origem a 2ª geração). Esses efeitos são considerados parentais, conduzindo a uma herança epigenética intergeracional. Somente a 3ª geração pode ser considerada com uma herança transgeracional verdadeira na ausência de exposição.

As alterações fenotípicas decorrentes de herança epigenética inter- ou transgeracional podem surgir por exposição a diversos os fatores ambientais com potencial para modulação de fatores epigenéticos, onde podemos destacar:

1. As influências *nutricionais* durante o período pré-natal, tais como a carência alimentar (HEIJMANS et al., 2008); o excesso de nutrientes (BYGREN; KAATI; EDVINSSON, 2001); uma dieta pobre em proteínas (CARONE et al., 2010) ou rica em gordura (NG et al., 2010), ou ainda uma dieta rica em compostos bioativos, como a genisteína (DOLINOY et al., 2006); a deficiência em vitaminas ou micronutrientes (MEJOS et al., 2013); a presença de toxinas derivadas do plástico (DOLINOY; HUANG; JIRTLE, 2007; MANIKKAM et al., 2013).
2. A influência do estilo de vida pré-natal, tais como o tabagismo (NORTHSTONE et al., 2014); o estresse (RODGERS et al., 2013); a obesidade (SOUBRY et al., 2013); e o exercício físico (LAKER et al., 2014).
3. Ainda a exposição a outros fatores durante o período pré-natal pode ser determinante para o fenótipo da prole, tais como a exposição a tintas e solventes (REID et al., 2011); pesticidas (SKINNER et al., 2013), radiações ionizantes (KOTURBASH et al., 2006); armas químicas (NGO; TAYLOR; ROBERTS, 2010); dentre outros.

A exposição a esses fatores durante o período pré-natal pode ter significativas implicações sobre as gerações futuras, tais como as alterações metabólicas

(obesidade, hipertensão, alteração em perfil lipídico, alteração na tolerância à glicose e redução da sensibilidade à insulina); doenças metabólicas (diabetes e doenças cardiovasculares); alterações psicossociais, comportamentais e mentais; prejuízo do desenvolvimento embrionário e crescimento fetal (com recém-nascidos de baixo peso e extremo baixo peso, ou com mal formações); e câncer (SOUBRY, 2015).

Nesse estudo avaliamos os efeitos da exposição durante o período gestacional a dois fatores ambientais: a suplementação materna com resveratrol - um composto bioativo encontrado na dieta; e a realização de exercício físico pela mãe – um componente do estilo de vida; sobre a toxicidade da DOX no coração da prole. A escolha desses fatores é relevante, pois tanto o resveratrol quanto o exercício físico possuem características cardioprotetoras, onde a sua utilização tem sido associada à proteção contra problemas cardíacos e doenças cardiovasculares (BERTELLI; DAS, 2009; MOORE, 1998; RAVAL et al., 2008). Exercício e resveratrol também apresentam como ponto comum atuarem sobre a redução do estresse oxidativo e aumento na resposta de sistemas de defesa antioxidante (ASCENSÃO et al., 2003; JI, 2002; UNGVARI et al., 2007). Esse efeito foi observado nos cardiomiócitos de neonatos provenientes de genitoras que realizaram exercício durante o período gestacional ou receberam suplementação com resveratrol na dieta, os quais apresentaram redução do nível de ERO e dano oxidativo ao DNA, bem como aumento na atividade de enzimas antioxidantes e expressão da CAT.

Relativamente ao exercício físico, sabe-se que sua adoção contribui de forma significativa para um estilo de vida saudável e conduz ao melhor prognóstico, redução do risco e dos efeitos colaterais relacionados ao tratamento medicamentoso de diversas doenças, incluindo o câncer, as doenças cardiovasculares, desordens metabólicas e doenças neurodegenerativas (LEE et al., 2012a; SCHMID; LEITZMANN, 2014). Além disso, o exercício físico é uma prática de baixo custo, acessível e que tem implicações sociais e econômicas positivas. Apesar de seus inúmeros benefícios, o completo conhecimento acerca de seus mecanismos moleculares ainda é limitado.

Durante a última década pesquisas na área da epigenética demonstraram que as modificações da cromatina, ou modificações epigenéticas, tais como modificações de

histonas e a metilação do DNA, apresentam um papel importante na patogênese de diversas doenças, dentre elas as doenças cardiovasculares. Além disso, estudos recentes nesse campo sugerem que o exercício pode agir como um potente modulador de modificações epigenéticas, e que possui potencial para conter o desenvolvimento e progressão de diversas doenças, como as cardiovasculares, em nível epigenético (CAROLINA et al., 2014; LOVATEL et al., 2013b; RECCHIONI et al., 2016; ZIMMER et al., 2015).

Em nosso estudo demonstramos que realização de exercício físico durante a gestação protege o coração da ninhada contra a toxicidade induzida pela DOX, onde foi observado um aumento significativo na viabilidade dos cardiomiócitos, com menor número de células em apoptose e necrose após o tratamento com a DOX, resultados que se correlacionaram com o decréscimo na produção de ERO. Nosso modelo de cardioproteção envolve o aumento da atividade do sistema de defesa antioxidante (aumento de atividade das enzimas SOD e CAT, bem como aumento da expressão da CAT) em resposta à presença de ERO, o que é frequentemente definido como um mecanismo de adaptação induzida pelo exercício (ATALAY; SEN, 1999; POWERS et al., 1998; RAMIRES; JI, 2001; VENDITTI; DI MEO, 1996).

De maneira significativa os resultados demonstraram que além dos efeitos sobre o sistema de defesa antioxidante, o exercício diminui o dano ao DNA no coração do neonato, através da redução no número de quebras duplas e danos álcali-lábeis, bem como das quebras simples indicativas de dano oxidativo, o qual é reconhecido pelas enzimas FPG e EndoIII. Podemos dizer que a redução do dano ao DNA pelo exercício durante a gestação ocorreu por um aumento no reparo dos danos, observado também nos cardiomiócitos não expostos ao quimioterápico. Esse dado indica que mecanismos de reparo podem ser ativados pelo exercício materno, reparando os danos basais nos cardiomiócitos da ninhada e possibilitando que a maquinaria de reparo esteja modulada para insultos adicionais, como pela exposição à DOX.

A preservação da integridade do DNA é essencial pra garantir uma herança precisa do material genético, bem como a adequada função celular; onde o dano ao DNA, não reparado, frequentemente conduz a senescência celular, apoptose ou

tumorigênese (PAPAMICHOS-CHRONAKIS; PETERSON, 2013). Inúmeros mecanismos têm evoluído no sentido de proteger e reparar o DNA danificado, tais como o reparo de quebras duplas por recombinação homóloga e não-homóloga (CHAPMAN; TAYLOR; BOULTON, 2012). Nesse processo inúmeros fatores estão envolvidos no reconhecimento, na sinalização e amplificação da cascata de reparo desencadeada pela formação de quebras na dupla fita do DNA (CICCIA; ELLEDGE, 2010). Dessa resposta orquestrada fazem parte inúmeras proteínas, dentre elas a Sirt6 que recebeu papel de destaque nesse trabalho por seu envolvimento em duas funções distintas, discutidas a seguir.

A Sirt6 é uma proteína nuclear membro da família das sirtuínas que apresenta uma função importante na estabilidade do genoma (MAO et al., 2011; TOIBER et al., 2013). Durante situações de estresse oxidativo, tais como o induzido pela DOX nos cardiomiócitos da prole, a Sirt6 é recrutada para os locais de quebra da dupla fita do DNA, onde atua sinalizando para o reparo do DNA por recombinação homóloga e não-homóloga. Nessa situação pode atuar como mono-ADP-ribosilase, situação em que a Sirt6 liga-se e mono-ADP-ribosila a PARP-1 (*Poly-ADP-ribose polymerase1*), ativando-a e aumentando o reparo do DNA (MAO et al., 2011); ou desacetilando a nuclease CtIP (*carboxy-terminal binding protein-interacting protein*), envolvida no processo de reparo de quebras duplas no DNA durante a recombinação homóloga (KAIDI et al., 2010).

Nosso trabalho demonstrou que o exercício materno protege o coração do neonato do dano ao DNA provocado pela exposição à DOX, proteção que está relacionada ao aumento na expressão da Sirt6, que pode ser recrutada para sítios de quebra do DNA amplificando a resposta ao dano. Entretanto, um efeito surpreendente do exercício materno durante a gestação foi o aumento na expressão da Sirt6 em cardiomiócitos e neonatos que não foram expostos à DOX, onde encontramos um efeito unicamente do exercício sobre a expressão da proteína. Para uma melhor compreensão desse resultado, recordamos que a Sirt6 é uma HDAC classe III dependente de $NAD^+$, coenzima cujos níveis encontram-se aumentados durante o exercício. Uma estreita relação entre os níveis da coenzima e da proteína já foi demonstrada no coração, quando se observaram níveis reduzidos de $NAD^+$ paralelamente à baixa atividade da Sirt6 durante a hipertrofia cardíaca (CAI et al.,

2012). Assim, considera-se que o aumento dos níveis de $NAD^+$ pelo exercício serve como um sensor metabólico para a ativação da Sirt6.

Tendo em vista que os resultados de cardioproteção no coração da ninhada foram obtidos por meio da intervenção realizada com exercício durante a gestação, outro aspecto importante a ser discutido é o relacionado aos efeitos das intervenções maternas sobre o fenótipo da prole. Nesse sentido, é importante destacar que a Sirt6 é uma HDAC que tem recebido destaque por seu papel em processos de modificação da cromatina, onde através das modificações pós traducionais de histonas atua em processos de modificação epigenética (KUGEL; MOSTOSLAVSKY, 2014; MOSTOSLAVSKY et al., 2006; TENNEN; CHUA, 2011).

As histonas possuem um papel central na organização da cromatina e acessibilidade dos genes. A conexão do DNA e das proteínas histonas é baseada em forças de atração eletrostática entre a carga negativa do DNA e a positiva da cadeia lateral de aminoácidos na porção N-terminal das histonas. Dessa forma, as modificações pos-traducionais de proteínas histonas na cadeia lateral de aminoácidos podem alterar a sua carga, modificando as forças de atração ou repulsão, e gerando um DNA que alterna entre um estado menos compactado e mais acessível ou mais compactado e inacessível, determinando ou não a criação de locais para recrutamento de proteínas ativadoras ou silenciadoras de genes alvo (BERGER et al., 2009; KOUZARIDES, 2007). Dentre as modificações de proteínas histonas que resultam em modificações estruturais na cromatina, com maior ou menor acessibilidade a fatores de transcrição e genes alvo, destacam-se a acetilação de resíduos de lisina das histonas pelas enzimas HATs, que deixam a cromatina num estado “ativo” e acessível; e a desacetilação de histonas, pelas HDACs, que faz o papel oposto, “silenciando” a cromatina ao reduzir a acessibilidade a fatores de transcrição (PAPAMICHOS-CHRONAKIS; PETERSON, 2013; WOLFFE, 1992).

Dessa forma, a Sirt6 além de atuar na sinalização para o reparo do DNA mantendo a integridade do genoma, desempenha uma função importante ao nível da cromatina, transmitindo seus sinais através da desacetilação de histonas. A Sirt6 recebe a denominação de reguladora trasnscricional, uma vez que altera a expressão

de inúmeros genes (KAWAHARA et al., 2009, 2011; TENNEN; CHUA, 2011). Dentre os alvos moleculares da Sirt6, atua desacetilando a histona H3 nos resíduos de lisina 9 e 56 (H3K9 e H3K56, respectivamente) e inibindo a atividade de diversos fatores de transcrição, como o NF-κB (factor nuclear kappa B), c-JUN (*Transcription factor AP-1*), HIF-1$\alpha$ (*Hypoxia-inducible factor 1-alpha*), e Myc-c (*Myc proto-oncogene protein*), envolvidos no processo de inflamação, envelhecimento, regulação do sistema cardiovascular, e câncer (KAWAHARA et al., 2009; MOSTOSLAVSKY et al., 2006; SEBASTIÁN et al., 2012; ZHONG; MOSTOSLAVSKY, 2010). Nesse sentido, estudos tem demonstrado que, através de sua função regulatória, a Sirt6 previne o desenvolvimento de hipertrofia e falência cardíaca, modula o metabolismo da glicose, inibe o processo inflamatório e a senescência de células endoteliais além de bloquear o crescimento tumoral (ANDERSON et al., 2015; CAI et al., 2012; CARDUS et al., 2013; SEBASTIÁN; MOSTOSLAVSKY, 2015). Também foi recentemente demonstrado que a desacetilação da H3K9 pela Sirt6 regula a ligação do fator de transcrição SFR (*serum-response factor*) ao DNA, controlando a diferenciação de células musculares lisas em cardiomiócitos e miócitos vasculares (MCDONALD et al., 2006; YAO et al., 2014).

Ao nível da cromatina, ainda são escassos os estudos que investigaram os efeitos do exercício sobre cardiomiócitos, tanto em humanos quanto em animais. Nessa linha, McGee e colaboradores demonstraram que uma única intervenção com exercício aeróbico em bicicleta ergométrica modifica o perfil da acetilação de histonas no músculo esquelético de adultos jovens (MCGEE et al., 2009). Além disso, a regulação epigenética pelo exercício foi demonstrada sobre a função endotelial, uma vez que o estresse de cisalhamento (“shear stress”) induzido pelo exercício pode modular a regulação da eNOS (óxido nítrico sintase endotelial) em nível transcricional, pela modulação da acetilação de histonas H3 e H4 (ILLI et al., 2003; WEBER et al., 2010).

Além disso, os efeitos intergeracionais do exercício realizado durante a gestação sobre a resistência do coração da prole a cardiotóxicos constitui um tópico ainda não investigado. Resultados que dão suporte para os efeitos protetores intergeracionais do exercício materno durante a gestação, demonstram em modelo experimental de camundongos expostos a dieta hipercalórica, que quando o exercício é realizado durante a gestação pode impedir que o PGC-1$\alpha$ (*peroxisome proliferator-activated*

*receptor gamma coactivator 1-alpha*) do músculo esquelético da prole seja hipermetilado, efeito normalmente observado no diabetes mellitus tipo 2 (LAKER et al., 2014).

Assim como o estilo de vida, a nutrição é um importante fator ambiental com capacidade de modular o fenótipo. Nessa linha, o resveratrol é um polifenol encontrado em pequenas quantidades na dieta que apresenta efeitos benéficos sobre a saúde, dentre eles a quimioprevenção contra o câncer e a proteção contra doenças cardiovasculares (DAS; DAS, 2007; WAFFO-TÉGUO et al., 2001). Nesse trabalho optamos pela suplementação materna durante a gestação com uma baixa dose de resveratrol (2,5 mg/Kg/dia), visto que não apresentou toxicidade, e esteve associada com efeito cardioprotetor em modelo experimental de animais hipertensos (MOVAHED et al., 2012). Com a administração dessa baixa dose de resveratrol, não observamos efeitos tóxicos para a mãe, como perda de peso durante a suplementação, ou para a prole, com redução do número de neonatos por ninhada. Além disso, tem sido demonstrado que existe uma relação não linear de resposta à dose de resveratrol para seus efeitos protetores tanto em humanos quanto em animais. Em modelo experimental animal de quimioprevenção foi comprovado que doses mais baixas estão associadas com melhores resultados, com redução de 52% no volume de adenoma coloretal numa baixa dose de resveratrol em comparação a 25% de redução do volume do tumor numa dose elevada (CAI et al., 2015).

Contudo, a administração de uma baixa dose não implica em ausência de efeitos sobre o feto, uma vez que o resveratrol pode atravessar a barreira placentária. Esse fato faz com que a cardioproteção observada em nosso modelo possa ser devida à ação direta da molécula, que possui ação antioxidante. Nesse sentido, Singh e colaboradores (SINGH et al., 2011) demonstraram que a suplementação materna com resveratrol durante a gestação (3º ao 12º dia gestacional) com 100 mg/Kg/dia previne o estresse oxidativo e a apoptose associadas com a embriopatia diabética, além de reduzir o perfil glicêmico e lipídico nas ratas prenhas. Embora a dose de resveratrol utilizada em nosso estudo seja baixa, a hipótese de um mecanismo de cardioproteção que envolva a ação direta da molécula não pode ser descartada.

Além disso, o resveratrol é um composto bioativo da dieta que tem demonstrado atuar também na modulação de fatores epigenéticos (CHOI; FRISO, 2010). Estudos anteriores demonstraram que o resveratrol pode inibir o crescimento de células tumorais com mutação em BRCA1 (*breast cancer 1*), por meio do aumento da expressão da Sirt1, que atuando juntamente com outros fatores resulta na inibição da cascata de sinalização do NFκB-p65 (*nuclear factor NF-kappa-B p65 subunit*) em linhagem de células de câncer de mama MCF-7 (*human breast adenocarcinoma cell line; acronym of Michigan Cancer Foundation-7*) (BOURGUIGNON; XIA; WONG, 2009; WANG et al., 2008). Outro efeito epigenético do resveratrol está relacionado ao seu papel no recrutamento da proteína MBD2 (*methyl-binding domain protein-2*) ao promotor do BRCA1 em linhagem de MCF-7 (PAPOUTSIS et al., 2010). A MBD2 é um membro da família de proteínas com domínio de ligação metil-CpG que apresenta papel importante no silenciamento epigenético de genes supressores tumorais.

Em nosso estudo verificamos que a suplementação materna com resveratrol durante a gestação causou um aumento significativo na expressão da Sirt6 nos cardiomiócitos de neonatos expostos à DOX. Como mencionado anteriormente, a Sirt6 é uma enzima nuclear com função importante na estabilidade do genoma, uma vez que é rapidamente recrutada para locais de quebra no DNA ativando fatores de reparo (CAI et al., 2012; MAO et al., 2011; TOIBER et al., 2013). Assim, entendemos que além da ação sobre o estresse oxidativo, outro mecanismo de cardioproteção pelo resveratrol está associado à redução do dano ao DNA, por meio do aumento na expressão Sirt6, a qual age como proteína reguladora na sinalização do reparo do dano ao DNA causado pela DOX.

Particularmente, o aumento na expressão da Sirt6 nos cardiomiócitos da ninhada de mães suplementadas com resveratrol foi dependente da concentração de DOX e não foi observado em cardiomiócitos de neonatos não expostos ao quimioterápico. O aumento na expressão da Sirt6 em cardiomiócitos de ratas suplementadas com resveratrol pode estar relacionado ao aumento nos níveis de $NAD^+$, uma vez que o resveratrol atua na mitocôndria inibindo a ATP sintase e ativando a AMPK, com consequente aumento dos níveis de $NAD^+$, o qual serve como um sensor metabólico para o aumento na expressão da Sirt6 (DOLINSKY et al., 2009; GLEDHILL et al., 2007;

HART et al., 2013). Relativamente ao aumento na expressão da Sirt6 pela exposição dos cardiomiócitos à DOX, entendemos que como a Sirt6 é considerada uma proteína de defesa, será acionada pelas células ao ativarem vias de sobrevivência celular. Esse mecanismo é observado quando ocorre aumento na expressão da Sirt6 em situações de estresse celular como o dano hipóxico ao coração (MAKSIN-MATVEEV et al., 2015), a hipertrofia cardíaca (CAI et al., 2012), a senescência prematura de condrócitos (NAGAI et al., 2015), e aqui podemos incluir a toxicidade cardíaca induzida pela DOX nos cardiomiócitos da prole, gerando dano ao DNA e instabilidade genômica, situações onde a Sirt6 será recrutada.

Assim, nossos resultados demonstram que o tanto o exercício realizado durante o período gestacional, quanto a suplementação materna com resveratrol durante a gestação apresentam efeitos cardioprotores intergeracionais sobre a toxicidade da DOX nos cardiomiócitos da ninhada. Durante a gestação a dieta é um dos principais fatores que influencia o desenvolvimento dos órgãos e a plasticidade do feto. Por esse motivo, o investimento em compostos bioativos da dieta que modulam diferentes mecanismos epigenéticos em diferentes estágios do desenvolvimento e podem agir de forma benéfica sobre a saúde e a susceptibilidade a diversas doenças no adulto, se faz importante. Da mesma forma o exercício tem efeitos reconhecidamente benéficos, e pode ser considerado uma ferramenta não-farmacológica promissora, usada para prevenir ou mesmo atenuar os efeitos colaterais provocados pelo tratamento de doenças graves, como o câncer.

Estudos adicionais são necessários para determinar os efeitos diretos de uma baixa dose de resveratrol, via barreira placentária, sobre o coração da prole; bem como o papel da Sirt6 sobre seus alvos moleculares (H3K9 e H4K56); da enzima PARP-1 e HATs nesse modelo de cardioproteção intergeracional pelo exercício e suplementação materna com resveratrol durante a gestação. Esses resultados irão possibilitar uma melhor compreensão dos efeitos do ambiente materno nas respostas a agentes estressores da prole no decorrer de seu desenvolvimento.

## 7. CONCLUSÕES

- ✓ O exercício ou a suplementação com resveratrol durante a gestação tornam o coração da ninhada mais resistente à morte celular pela DOX;
- ✓ A apoptose é o principal mecanismo de morte celular induzida pela DOX nos cardiomiócitos de ninhadas de todos os grupos de tratamento;
- ✓ A cardiotoxicidade da DOX está relacionada ao aumento no estresse oxidativo nos cardiomiócitos das ninhadas;
- ✓ O exercício ou a suplementação com resveratrol durante a gestação reduzem significativamente o dano ao DNA induzido pela DOX nos cardiomiócitos da ninhada;
- ✓ O exercício ou a suplementação com resveratrol durante a gestação aumentam a capacidade de defesa antioxidante dos cardiomiócitos das ninhadas;
- ✓ A HDAC Sirt6 tem expressão aumentada em cardiomiócitos de ninhadas de genitoras que realizaram exercício durante a gestação;
- ✓ Existem efeitos intergeracionais cardioprotetores no coração da prole de exposições feitas às genitoras durante o período gestacional.

## 8. PERSPECTIVAS

As principais perspectivas para a continuidade deste trabalho, bem como para o desenvolvimento de outros estudos são apresentadas a seguir:

- Estimar os efeitos diretos do resveratrol no coração da ninhada, através da quantificação do resveratrol e seus metabólitos no coração do neonato;
- Avaliar a expressão dos alvos moleculares da Sirt6 – H3K9 e H3K56;
- Investigar adicionais mecanismos de reparo dos danos ao DNA, por meio de enzimas de reparo de quebras simples (PARP-1 e OGG1), ou de quebras duplas (Rad51, Ku70);
- Avaliar a expressão e atividade da Sirt1, que atua conjuntamente com a PARP-1, bem como a atividade da Sirt6;
- Estimar o nível de $NAD^+$, ATP e AMP intracelular, e sua relação com a expressão da Sirt6;
- Avaliar a expressão de proteínas relacionadas à metilação do DNA (MBP), bem como de histonas metiladas.

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# 10. ANEXOS

## 10.1. Parecer consubstanciado – CEUA/UFCSPA

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

**CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS**

**PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO**

**1) PROTOCOLO Nº: 106/13** **Parecer 182/13**

**2) DATA DO PARECER: 08/05/2013**

**3) TÍTULO DO PROJETO:**

Efeitos do exercício físico durante o período pré-natal sobre a cardiotoxicidade da DOX: avaliação da memória celular de cardiomiócitos de ratos neonatos

**4) PESQUISADOR RESPONSÁVEL:**

Dra Jenifer Saffi

**5) SUMÁRIO DO PROJETO:**

**6) OBJETIVOS DO PROJETO:**

Objetivo Geral:
Verificar os efeitos do exercício físico realizado durante o período pré-natal e gestacional sobre a cardiotoxicidade da DOX, as alterações na cromatina e formação de memória celular nos cardiomiócitos de ratos neonatos

**7) FINALIDADE DO PROJETO:** [ ] Ensino [X] Pesquisa

**8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:**

| | | |
|---|---|---|
| **Título** | [X] Adequado | [ ] Comentários |
| **Introdução** | [X] Adequada | [ ] Comentários |
| **Objetivos** | [X] Adequados | [ ] Comentários |
| **Relevância e Justificativa** | [X] Adequados | [ ] Comentários |
| **Materiais e Métodos** | [X] Adequados | [ ] Comentários |
| **Cronograma para execução da pesquisa** | [X] Adequado | [ ] Comentários |
| **Orçamento e fonte financiadora** | [X] Adequados | [ ] Comentários |
| **Referências Bibliográficas** | [X] Adequadas | [ ] Comentários |

**9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:** [X] Sim [ ] Não

REPÚBLICA FEDERATIVA DO BRASIL
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UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

**10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:**

**Grau de dor/estresse:** B [x] C [x] D [ ] E [ ]

**Espécie:** Wistar **Número Amostral:** 80

| | | |
|---|---|---|
| **Redução Amostral:** | [ ] Sim | [x] Não |
| **Substituição de Metodologia:** | [ ] Sim | [x] Não |
| **Aprimoramento da Metodologia:** | [ ] Sim | [X] Não |
| **Acomodação e manutenção dos animais:** | [X] Adequada | [ ] Inadequada |
| **Manipulação dos animais:** | [X] Adequada | [ ] Inadequada |
| **Analgesia dos animais** (se aplicável) | [x] Adequada | [ ] Inadequada |
| **Anestesia dos animais** (se aplicável) | [x] Adequada | [ ] Inadequada |
| **Eutanásia dos animais** (se aplicável) | [X] Adequada | [ ] Inadequada |

**Local de Realização** (Biotério/Labotarório): Laboratório de Genética Toxicológica e Fisiologia da UFCSPA

Outra instituição: Não

**11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS**

| Data | Espécie | Sexo | Quantidade |
|---|---|---|---|
| Jul-2013-jul-2015 | wistar | machos | 20 |
| Jul-2013-jul-2015 | wistar | machos | 60 |
| Jul-2013-jul-2015 | wistar | Neonatos | Todos |

**12) RECOMENDAÇÃO:**

[x] Aprovado

[ ] Com Pendência

[ ] Não aprovado

**Comentários gerais sobre o projeto:**

10.2. Critérios para submissão de artigos ao periódico Toxicology.

# TOXICOLOGY

Affiliated with the German Toxicology Society

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AUTHOR INFORMATION PACK

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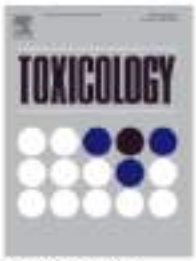


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Toxicologists, Pharmacologists, Epidemiologists, Teratologists.

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