Carolina Foot Gomes de Moura

AÇÃO DO CONCENTRADO DE SUCO DE UVA E DE SUCO DE MAÇÃ EM MÚLTIPLOS ÓRGÃOS DE RATOS EXPOSTOS AO CLORETO DE CÁDMIO

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, para obtenção do Título de Doutora em Ciências

São Paulo

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Orientador: Prof. Dr. Daniel Araki Ribeiro

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de Moura, Carolina Foot Gomes

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AÇÃO DO CONCENTRADO DE SUCO DE UVA E DE SUCO DE MAÇÃ EM MÚLTIPLOS ÓRGÃOS DE RATOS EXPOSTOS AO CLORETO DE CÁDMIO

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DEDICATÓRIA

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EPÍGRAFE

Deixe o alimento ser tua medicina e a medicina ser teu alimento.

Hipócrates

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

% – por cento

± – mais ou menos

® – Marca Registrada

µ – micrograma

µg/g – micrograma(s) por grama

µg/kg – micrograma(s) por quilograma

µL – microlitro

µm – micrometro(s)

µM – microMol

4-NqO – 4-nitroquinolina 1-óxido

8-OHdG – 8-hidroxi-20-guanosina

8-OHGua – 8-hidroxiguanina

ANOVA – análise de variância

BAL – dimercaprol

CAT – catalase

Cd – grupo cádmio

Cd+SM – grupo cádmio associado ao tratamento com suco de maçã

Cd+SU – grupo cádmio associado a tratamento com concentrado de suco de uva G8000®

cDNA – ácido desoxirribonucléico complementar

CEDEME – Centro de Desenvolvimento de Modelos Experimentais

CEUA – Comissão de Ética no Uso Animal

COX-2 – cicloxigenase tipo 2

CTRL – grupo controle

DAB – 3,3-diaminobenzidina

DEPC – dietilpirocarbonato

DFOA – desferrioxamina

DMSA – ácido meso 2,3-dimercaptosucínico

DNA – ácido desoxirribonucleico

DNAse – desoxiribonuclease

EDTA – etileno-diamino-tetracético

eNOS - óxido nítrico sintase endotelial

EROs – espécies reativas de oxigênio

EUA – Estados Unidos da América

g – grama(s)

g/dia – grama(s) por dia

g/kg – grama(s) por quilograma

GAE g/kg – equivalente de ácido gálico por quilograma

GAPDH – gliceraldeído trifosfato desidrogenase

H_2O_2 – peróxido de hidrogênio

IARC – Agência Internacional de Pesquisa sobre o Câncer

iNOS – óxido nítrico sintase induzido

ip – intraperitoneal

kg – quilograma

LDL - lipoproteínas de baixa densidade (*Low Density Lipoproteins*)

M – Mol

mA – miliAmpere

mg – miligrama(s)

mg/g – miligrama(s) por grama

mg/kg – miligrama(s) por quilograma

mg/L – miligrama(s) por litro

mL – mililitro(s)

mM – miliMol

MT – metalotioneínas

n – número de animais por grupo

NF-κB – fator nuclear kappa B

nm – nanômetro

nM – nanoMol

O_2^- – ânion superóxido

O_2 – oxigênio

^oC – graus Celsius

OMS – Organização Mundial da Saúde

PBS – tampão fosfato de sódio

PCR – cadeia de polimerase reversa

q.s.p. – quantidade suficiente para

qPCR - cadeia de polimerase reversa em tempo real

RNA – ácido ribonucleico

rpm – rotações por minuto

SOD – superóxido dismutase

SOD-CuZn – superóxido dismutase cobre-zinco

SOD-Mn – superóxido manganês

TGI – trato gastrointestinal

TNBS – ácido 2,4,6-trinitrobenzeno-sulfônico

TNF-α – fator de necrose tumoral alfa

Tris-HCL – hidrocloreto hidroximetil-aminometano

Triton X-100 – 4-(1,1,3,3-tetrametilbutil)fenil-poetileno glicol

UNIFESP – Universidade Federal de São Paulo

V – Volts

Vitamina C eq/kg – equivalente de Vitamina C por quilograma

RESUMO

O cádmio é um importante poluente industrial e ambiental devido à sua alta toxicidade. Este metal é pobremente excretado acumulando-se em vários órgãos, sendo responsável pelo desenvolvimento de inúmeras doenças. A prevenção a partir da dieta surgiu como uma nova alternativa para o tratamento dessas doenças; e diversos agentes nutracêuticos têm sido explorados na terapia contra os efeitos tóxicos de metais pesados. Neste estudo, avaliou-se o potencial efeito protetor dos concentrados de suco de uva G8000® e do suco de maçã contra a toxicidade induzida pelo cádmio em múltiplos órgãos de ratos. **MATERIAL E MÉTODOS**: ratos machos Wistar foram aleatoriamente distribuídos em quatro grupos, com cinco animais cada, conforme descrito a seguir: Grupo Controle (controle negativo – injeção intraperitoneal (ip) de água e, após 15 dias, administração de 1 mL de água por 15 dias, via gavage); Grupo cádmio (injeção ip de 1,2 mg/kg de cloreto de cádmio e, após 15 dias, administração de 1 mL de água por 15 dias, via gavage); Grupo cádmio + Suco de Uva (injeção ip de 1,2 mg/kg de cloreto de cádmio e, após 15 dias, administração de 0,8 mL de de concentrado de suco uva G8000® durante 15 dias, via gavage); Grupo cádmio + Suco de Maçã (injeção ip de 1,2 mg/kg de cloreto de cádmio e, após 15 dias, administração de 1 mL de concentrado de suco de maçã durante 15 dias, via gavage). **RESULTADOS**: Os animais expostos ao cádmio e tratados com o concentrado de suco de uva G8000® ou com suco de maçã apresentaram significativa diminuição no número de micronúcleos, menor quebra de fita de DNA (teste do cometa) e redução do estresse oxidativo (teste do desafio e imunoexpressão de 8-OHdG), quando comparados aos animais expostos somente ao cádmio. A análise de expressão gênica apontou que o concentrado de suco de uva G8000® elevou a expressão de SOD-CuZn e o suco de maçã foi capaz de alterar a expressão das enzimas SOD-CuZn, SOD-Mn e catalase. **CONCLUSÃO**: Em suma, nossos resultados revelam um elevado potencial hepatoprotetor, antigenotóxico, antimutagênico e antioxidante do concentrado de suco de uva G8000® e de suco de maçã, provavelmente devido à presença de seus compostos polifenólicos.

Palavras-chave: cádmio, suco de uva, suco de maçã, toxicidade.

ABSTRACT

Cadmium is one of the most important toxic environmental and industrial pollutants due to its high toxicity. This metal is poorly excreted accumulating in various organs being responsible for developing numerous diseases. Prevention from the diet has emerged as a new alternative for treating diseases; and several nutraceuticals agents have been used against toxic effects induced by heavy metals. In this study, we evaluated the potential protective effect of grape juice concentrate G8000™ and apple juice against toxicity induced by cadmium in multiple organs of rats. **MATERIAL AND METHODS**: male Wistar rats were ramdonly distributed into four groups, with five animals each, as follows: Control group (negative control – intraperitoneal (ip) water injection and, after 15 days, it was given 1 mL of water for another 15 days, via gavage); Cadmium group (single ip injection of cadmium chloride (1.2 mg/kg body weight), and after 15 days, it was given 1 mL of water for 15 days, via gavage); Grape Juice + cadmium group (single ip injection of cadmium chloride (1.2 mg/kg body weight), and after 15 days, it was given 0.8 mL of grape juice concentrate G8000™ for 15 days, via gavage) and Apple Juice + cadmium group (single ip injection of cadmium chloride (1.2 mg/kg body weight), and after 15 days, it was given 1 mL of apple juice for 15 days, via gavage). **RESULTS**: The groups exposed to cadmium and treated with grape juice concentrate G8000™ or apple juice had a significant decrease in the frequency micronucleated cells and DNA strand breaks (comet assay) as well as reduction in oxidative stress (challenge assay and 8-OHdG expression) when compared to animals exposed to cadmium only. Grape juice concentrate G8000™ increased CuZn-SOD expression and apple juice was able to modulate CuZn-SOD, Mn-SOD and catalase expression of this enzyme. **CONCLUSION**: Taken together, our results show a hepatoprotective, antigenotoxic, antimutagenic and antioxidant of grape juice concentrate G8000™ and apple juice, probably due to their polyphenolic compounds.

Keywords: cadmium, grape juice, apple juice, toxicity

1 INTRODUÇÃO

Nos últimos anos, a preocupação com a poluição ambiental e seus impactos na saúde tem sido objeto de diversos estudos (Hectors et al. 2011; Yi et al. 2011; Bollati e Baccarelli 2010; Perera et al. 2003). Evidências mostram que o desenvolvimento da indústria e da agricultura tem resultado em aumento da poluição e, consequentemente, em uma maior exposição a poluentes ambientais que, por sua vez, estão relacionados com a etiologia e patogênese de diversas doenças crônicas (Yi et al. 2011; Hennig et al. 2007).

Segundo a Organização Mundial da Saúde (OMS), a estatística mundial aponta que 23% de todas as mortes prematuras são atribuídas à contaminação por poluentes ambientais (Prüss-Üstün e Corvalán 2006). Os contaminantes ambientais são capazes de promover interações com o material genético resultando em alterações estruturais do DNA e, por conseguinte, mutações que podem levar à carcinogênese ou mesmo à morte celular (Gavina et al. 2014). Tais contaminantes podem ser encontrados no ar, água, solo e alimentos (Boffetta 2006). Alguns são de especial destaque, tais como efluentes de esgoto, derivados de petróleo, produtos decorrentes da mineração de aço e da conversão de carvão, metais pesados, plásticos, detergentes, pesticidas, produtos fenólicos, álcool, fumaça de cigarro, poluição urbana, entre outros (Wasi et al. 2013; Rappaport 2012)

Metais pesados como cádmio, cobre, ferro, níquel e zinco fazem parte da poluição ambiental e a prolongada exposição pode acarretar efeitos deletérios à saúde humana (Singh et al. 2011). Tem sido estabelecido que a intoxicação por metais pesados seja responsável por alterações em glândulas, sistema nervoso, coração, pulmões, fígado, rins, etc, assim como deflagrar diversas doenças crônicas (Caussy et al. 2003).

Vários metais e metalóides são classificados como cancerígenos pela Agência Internacional de Pesquisa sobre Câncer (IARC) (Ziech et al. 2010; Mena et al. 2009). O potencial de diferentes metais na geração de espécies reativas de oxigênio (EROs) é considerado como o principal mecanismo de carcinogênese induzido por metais, uma vez que alteram o mecanismo redox em células eucarióticas (Lee et al. 2012).

Diversos estudos demonstraram um aumento na incidência de cânceres associados à exposição crônica a metais pesados (Arita e Costa 2009).

O cádmio é um metal pesado altamente tóxico, podendo ser encontrado em solo, água, ar, alimentos e na fumaça liberada pelo cigarro. A dieta é a principal fonte de exposição ao cádmio ambiental em não-fumantes, e presente em praticamente quase todo tipo de alimento oriundo de regiões contaminadas (Engström et al. 2011; Satarug et al. 2010; Jarup e Akesson 2009; Panjehpour e Bayesteh 2008; Brzóska et al. 2003). Ele promove degeneração e subsequente morte celular em fígado, rins e testículo, além de hipertensão, aterosclerose, osteoporose, anemia e câncer (Abdelaziz et al. 2013).

Tendo em vista a extensa exposição humana ao cádmio, seja por meio da contaminação ocupacional, seja por meio de alimentos contaminados, aliado ao hábito de fumar, estudos que demonstrem a eficácia de compostos alimentares para mitigar os danos são bem-vindos e necessários, já que medidas preventivas em muitas situações são praticamente impossíveis.

A prevenção a partir da dieta surgiu como uma nova alternativa para o tratamento de diversas doenças crônicas, tais como doenças cardiovasculares, disfunções renal e pulmonar, danos gastrointestinais, doenças neuro-degenerativas e câncer (Del Rio et al. 2013; Bishayee et al. 2011; Nordberg 2009; Arora et al. 2008; Borges et al. 2008).

A compreensão dos mecanismos de ação envolvendo compostos com potencial nutracêutico tem sido abordada observando-se possíveis terapias contra os efeitos tóxicos de metais pesados como, por exemplo, o cádmio (Lawal e Ellis 2011). Biologicamente, tais metais atuam em várias vias de sinalização metabólicas (Flora et al. 2013) e sua potencial toxicidade é decorrente, principalmente, do estresse oxidativo (Nwokocha et al. 2012).

Uma ampla variedade de substâncias age contra radicais livres geradores de danos oxidativos e substâncias antioxidantes retardam ou mesmo inibem tal prejuízo (Sen et al. 2010). Resultados obtidos por Ognjanović et al. (2003) e El-Demerdash et al. (2004) ao associarem substâncias antioxidantes contra a ação nociva do cloreto de cádmio levaram a comunidade científica a investigar mecanismos que reduzam os

efeitos danosos deste metal e que sejam de fácil acesso à população, por meio da alimentação saudável a partir de compostos com potencial nutracêutico.

2 REVISÃO DE LITERATURA

2.1 Cádmio

O cádmio é um importante poluente industrial e ambiental (Prozialeck et al. 2009) devido à sua alta toxicidade. Ele é pobremente excretado acumulando-se em vários órgãos, sendo responsável por sérios danos em pulmões, cérebro, testículos, rins, fígado, sangue e ossos (El-Refaiy e Eissa 2013; Anetor 2012).

Alimentos contaminados representam uma das fontes de exposição ao cádmio ambiental oriundos de regiões contaminadas (Engström et al. 2011; Satarug et al. 2010; Jarup e Akesson 2009; Panjehpour e Bayesteh 2008; Brzóska et al. 2003). De acordo com Nasreddine e Parent-Massin (2002), 1/3 do consumo de cádmio por meio da dieta é advindo da ingestão de produtos de origem animal e 2/3, a partir de vegetais contaminados. A OMS, por sua vez, afirma que a ingestão diária humana de cádmio varia de 40 µg em regiões não poluídas a 200 µg para áreas contaminadas (Ivanova et al. 2013). Além disso, o tabagismo aumenta a exposição ao cádmio em fumantes ativos e passivos (Satarug 2012), uma vez que cada cigarro contém de 1 a 2 µg de cádmio e 40 a 60% do cádmio inalado é absorvido pelo sistema circulatório (Zalups e Ahmad 2003).

O contato com o cádmio causa intoxicação em vários tecidos humanos. A intoxicação aguda promove danos, principalmente, no fígado e aparelho reprodutor masculino, enquanto que a exposição crônica resulta em lesões renais e ósseas sendo responsável por diversas doenças como disfunção renal, prejuízo nos parâmetros reprodutivos e desordens esqueléticas, além de doenças cardiovasculares, pulmonares e do trato gastrintestinal (TGI), incluindo fígado e glândulas salivares. Não obstante, este metal ainda promove o desenvolvimento de cânceres, sendo classificado como agente cancerígeno pertencente ao grupo I (Nordberg 2009; Arora et al. 2008; Borges et al. 2008).

Estudos revelaram que o cádmio, após ser absorvido pela via pulmonar ou TGI, é transportado pela corrente sanguínea ligado à albumina (Nordberg 2009; Sánchez-González et al. 2006). Quando absorvido pelo fígado, inibe enzimas hepáticas

promovendo aumento da peroxidação lipídica, congestão, isquemia e hipóxia (Ramesh e Satakipan 2010). Concomitantemente, induz a síntese de metalotioneínas (MT), uma cadeia de aminoácidos que apresenta um sítio de ligação para metais. As MT se ligam ao cádmio e este complexo é filtrado pelos glomérulos renais e reabsorvidos pelos túbulos proximais, ao passo que enzimas digestivas degradam a parte proteica deste complexo e os íons de cádmio liberados reiniciarão a síntese de MT. Quando os níveis de cádmio livre superam a produção de MT, ocorrem danos nas membranas celulares renais e, consequentemente culminam na insuficiência renal (Zhao et al. 2010; Sánchez-González et al. 2006).

Nos pulmões, a absorção e o acúmulo de cádmio são responsáveis pelo desenvolvimento de doenças como enfisema, bronquite e câncer (Ezzat et al. 2009; Panjehpour e Bayesteh 2008). A toxicidade pulmonar por metais pesados está relacionada com a presença de infiltrado leucocitário e seus efeitos pró-inflamatórios (Kataranovsky et al. 2009). Zhao e colaboradores (2010) afirmaram que ocorre um aumento de MT também nos pulmões como resposta de defesa do organismo frente à presença de cádmio no tecido.

Czykier et al. (2003) estudaram os efeitos da exposição crônica ao cádmio em ratos e os resultados mostraram que a concentração do metal em glândulas submandibulares é dose-dependente. Friedrichi et al. (2009) afirmaram que o cádmio é transferido em maior concentração para ratos recém-nascidos durante a lactação em detrimento da gestação; promovendo, assim, um retardo no crescimento e uma menor diferenciação das glândulas parótida, submandibular e sublingual.

Pesquisas demonstraram que o cádmio também possui efeito osteotóxico. Schutte e colaboradores (2008) estudaram a relação entre reabsorção óssea e exposição ao cádmio ambiental e concluíram que este metal aumenta a reabsorção óssea em mulheres. Resultados semelhantes foram obtidos por Engström et al. (2011) e Nawrot et al. (2010) ao avaliarem a associação entre o cádmio e a densidade mineral óssea em mulheres e homens. Tais efeitos devem-se ao fato do cádmio afetar o comportamento dos osteoblastos interferindo na sua maturação, assim como na expressão de marcadores bioquímicos relacionados à remodelação óssea (Bodo et al. 2010).

Independentemente do tecido afetado, a toxicidade desencadeada pelo cádmio é inicialmente caracterizada por danos oxidativos (Eybl et al. 2006). Estudos têm sugerido que o mecanismo de toxicidade aguda induzida por cádmio envolve a depleção de glutationa e de proteínas ligadas ao grupo das sulfidrilas, resultando no aumento de espécies reativas de oxigênio que, por sua vez, promove a peroxidação lipídica e em última instância, danos ao DNA (El-Refaiy e Eissa 2013; Liu et al. 2009).

2.2 O papel de agentes nutracêuticos perante a intoxicação por metais pesados

Diversos agentes nutracêuticos têm sido explorados na terapia contra os efeitos tóxicos de vários compostos, incluindo metais pesados. Tais substâncias exercem sua função por meio da indução de enzimas detoxificantes promovendo reações de oxidação, redução, hidrólise ou conjugação, a fim de diminuir ou eliminar a toxicidade induzida no organismo (Lawal e Ellis 2011).

A toxicidade de muitos metais ocorre por meio de danos gerados por EROs (Bower et al. 2005). Biocompostos antioxidantes têm apresentado a capacidade de inativar tais compostos, responsáveis pela iniciação de diversas doenças (Matés et al. 2013). Grandes esforços têm sido feitos na tentativa de encontrar seguros e potentes agentes antioxidantes de origem vegetal (Ramesh e Satakipan 2010). Além de vitaminas, diversas substâncias potencialmente antioxidantes têm sido estudadas em animais expostos aos metais pesados.

Os polifenóis consistem na maior classe de antioxidantes. Os flavonóides representam os polifenóis mais abundantes na dieta humana, compreendendo milhares de compostos (de Moura et al. 2013). Biologicamente, um dos mecanismos de ação atribuídos aos polifenóis é relacionado à remoção do metal bem como sequestro de radicais livres (Flora et al. 2013; Fraga et al. 2010). A inativação destes elementos ocorre quando um antioxidante interage com o radical livre mesmo quando a reação conduz à formação de outro radical, porém menos reativo (Leopoldini et al. 2011).

Vários compostos naturais têm sido descritos na literatura como quelantes por serem hábeis na remoção de metais tóxicos, formando um complexo estável, mobilizando metais nos tecidos e mantendo a porção quelato durante a circulação aos rins (para excreção na urina) e fígado (para excreção na bílis) (Flora et al. 2013; Sears 2013).

Estudos realizados para avaliar a ação de antioxidantes na prevenção de instabilidade genômica induzida por cádmio foram motivados por diversos autores (Anetor 2012; Ramesh e Satakopan 2010) ao acreditarem que o uso de compostos antioxidantes naturais presentes na dieta seria uma forma relativamente simples de atenuar os danos causados pela intoxicação induzida por metais pesados (Renugadevi e Prabu 2010).

2.3 Suco de Uva

Tendo em vista que a reversão dos danos induzidos pelo cádmio é difícil, a utilização de agentes nutracêuticos parece ser uma estratégia popular e relativamente fácil para proteger os seres humanos contra o risco de sérios problemas de saúde em caso de exposição a metais tóxicos.

O resveratrol é um polifenol considerado o maior composto bioativo das fitoalexinas estilbeno, sintetizado por folhas de videiras em resposta à infecção fúngica ou a exposição à luz ultravioleta na forma trans-resveratrol (Frémont 2000). No organismo, atua como antioxidante reduzindo a formação de EROs, modula o metabolismo de lipídeos e lipoproteínas, inibe a agregação plaquetária, possui ação anti-inflamatória, principalmente pela inibição da expressão gênica do Fator Nuclear kappa B (NF-κB), redução da proliferação celular e desencadeamento de apoptose em células tumorais (Frémont 2000; Benitez et al. 2009; Colin et al. 2009; Luna et al. 2009). Segundo Frémont (2000), o tempo de fermentação em contato com a casca é determinante na concentração de polifenóis presentes no vinho, uma vez que o resveratrol é produzido pela casca e não pela polpa da uva. Sendo assim, o vinho branco possui baixa quantidade de compostos polifenólicos devido ao curto tempo de maceração necessário para sua produção.

Em um trabalho de revisão de literatura, Maydata (2002) relatou a relação inversa entre o consumo de polifenólicos presentes no vinho e a ocorrência de doenças cardiovasculares, principalmente por evitar a oxidação da fração LDL (lipoproteínas de baixa densidade, do inglês *Low Density Lipoproteins*) de colesterol, mesmo na fase pós-prandial imediata. O suco de uva também conferiu proteção contra lesões oxidativas, embora sua concentração de polifenólicos seja a metade da encontrada no vinho (Maydata 2002).

Gollücke et al. (2010) descreveu em trabalho de revisão de literatura a significativa melhora no metabolismo de lipoproteínas, do estresse oxidativo e de marcadores inflamatórios bem como inibição da agregação plaquetária como alguns efeitos benéficos dos polifenóis e antocianinas presentes na uva. É importante ressaltar que esses polifenóis sofrem diversas reações químicas durante o processamento e armazenamento, incluindo polimerização e despolimerização, reações enzimáticas e co-pigmentação. Entretanto, tais transformações não afetariam necessariamente o conteúdo final e a atividade antioxidante dos mesmos (Gollücke et al. 2010).

Em parâmetros reprodutivos, Jiang et al. (2008) identificaram aumento do peso testicular e melhora na morfologia dos túbulos seminíferos de ratos expostos a 2,4-hexanediona e tratados com resveratrol, embora ainda apresentassem diferenças significativas em relação ao controle. Além disso, Juan et al. (2005) observaram manutenção do peso testicular, aumento da contagem espermática e nos níveis séricos do hormônio folículo estimulante, hormônio luteinizante e testosterona em ratos saudáveis tratados com resveratrol.

Nos últimos anos, nosso grupo de pesquisa investe em análises acerca dos efeitos benéficos do suco de uva em diversos modelos experimentais. Em animais tratados com dieta hiperlipídica, o suco de uva preveniu os danos oxidativos no sangue periférico gerados pela dieta rica em colesterol (Aguiar Jr. 2011). No modelo experimental de colite induzida por TNBS (ácido 2,4,6-trinitrobenzeno-sulfônico), o suco de uva atenuou danos histopatológicos, tais como ulceração e necrose causados pelo TNBS (Paiotti et al. 2013) além de modular a atividade anti-inflamatória, diminuindo a imunoexpressão de COX-2 (cicloxigenase tipo 2), iNOS (óxido nítrico sintase induzido) e TNF-α (fator de necrose tumoral alfa) (Marchi et al. 2014). No

estudo de carcinogênese bucal induzida por 4-NqO (4-nitroquinolina 1-óxido), o suco de uva reduziu lesões hiperplásicas e displásicas promovidas pelo agente cancerígeno, diminuiu a expressão gênica de COX-2, TNF-α e eNOS (óxido nítrico sintase endotelial), elevou a expressão dos genes antioxidantes SOD-CuZn (superóxido dismutase cobre-zinco) e catalase (CAT), além de suprimir a expressão do marcador de proliferação celular ki-67 (de Jesus et al. 2014).

No que tange ao sistema reprodutor, nosso grupo participou de estudo que investigou os efeitos do suco de uva em ratos intoxicados pelo cádmio. Para tanto, duas doses de suco de uva (1,18 e 2,36 g de polifenóis por kg) foram utilizadas para avaliar parâmetros reprodutivos, cujos resultados demonstraram que, na maior concentração, o suco de uva amenizou os danos causados pelo cádmio, melhorando os níveis de testosterona, o peso relativo do epidídimo e da próstata e a porcentagem de espermatozóides com morfologia normal (Pires et al. 2013). Além disso, o suco de uva nessa concentração normalizou os níveis da atividade enzimática da SOD (superóxido dismutase) e glutationa; confirmando os seus efeitos benéficos perante os danos causados pelo cádmio (Pires et al. 2013). Dessa forma, seria interessante avaliar se o suco de uva também é apto a modular os efeitos deletérios induzidos pelo cádmio em outros tecidos e órgãos relacionados direta ou indiretamente ao metabolismo, tais como sangue, fígado e rim.

2.4 Suco de Maçã

A maçã (*Malus sp*) possui alta concentração de polifenóis e outros fitoquímicos distribuídos, principalmente, pela casca, núcleo e polpa, em uma proporção de 110 a 357 mg de polifenóis por 110 g de fruto fresco (Lata 2007; Wolfe et al. 2003), sendo rica fonte de flavonóides, catequinas, antocianinas, diidrocalcones, ácido fenólico e procianidinas (Peterman et al. 2009; van der Sluis et al. 2005; Boyer e Liu 2004). Dentre a classe de favonóides, a maçã apresenta alta concentração de quercetina glicosilada que, para ser absorvida pelo organismo, é hidrolisada a quercetina e convertida a quercetina glucuronida e sulfatos no organismo humano (van der Sluis et al. 2001). Na maçã, a quercetina é encontrada na casca do fruto (1mg/g de peso do fruto fresco); enquanto que, o fruto não apresenta nenhum outro flavonóide quando descascado (Scalbert e Williamson 2000). Além destes compostos, a maçã possui

componentes não nutrientes, tais como fibra alimentar, minerais e vitaminas (Gerhauser 2008).

Diversos estudos demonstraram que os polifenóis presentes na maçã apresentam atividade antioxidante, inibem a proliferação celular e induzem apoptose; características estas que parecem estar envolvidas com o mecanismo de quimioprevenção (Peterman et al. 2009) e podem contribuir também em doenças cardiovasculares, respiratórias, diabetes e obesidade (Gerhauser 2008). Hyson (2011) relatou que o consumo de uma ou mais maçãs (166 g/dia) reduz o risco de câncer oral e de faringe em 18%, esôfago (22%), coloretal (30%), laringe (41%), mama (24%), ovário (24%) e próstata (7%). Outros autores confirmaram tais achados (McCann et al. 2007; Gallus et al. 2005; Gossé et al. 2005; Boyer e Liu 2004; Wolfe et al. 2003).

Na carcinogênese bucal experimental murina, nosso grupo de pesquisa demonstrou que o suco de maçã diminuiu a incidência de lesões hiperplásicas e displásicas além de reduzir a expressão gênica de COX-2 e TNF-α e aumentou a expressão de citocromo C e caspase-3; ratificando que o suco de maçã suprime a carcinogênese no que tangue à sua ação antioxidante e atividade apoptótica (Ribeiro et al. 2014).

Considerando os benefícios de substâncias antioxidantes e sua eficácia em reduzir os danos causados pelo cádmio, este trabalho avaliou os efeitos do concentrado de suco de uva e do suco de maçã, ricos em polifenóis em sangue e fígado de roedores submetidos à intoxicação aguda por cádmio.

3 OBJETIVO

O objetivo do presente estudo consistiu em avaliar os efeitos do concentrado de suco de uva e suco de maçã em múltiplos órgãos de roedores expostos ao cloreto de cádmio. Para tanto, foram avaliados os seguintes parâmetros:

- estresse oxidativo em fígado
- danos mutagênicos e genotóxicos em tecido hepático e em sangue periférico
- danos oxidativos genômicos e a capacidade de reparo em tecido hepático
- expressão gênica de enzimas antioxidantes em fígado.

4 MATERIAL E MÉTODOS

4.1 Animais

Foram utilizados 20 ratos machos Wistar, adultos (90 dias), provenientes do Centro de Desenvolvimento de Modelos Experimentais (CEDEME) da Universidade Federal de São Paulo (UNIFESP), e mantidos na guarda de animais da Universidade Federal de São Paulo *Campus* Baixada Santista, em temperatura média 22ºC ± 2 ºC, ciclo claro/escuro de 12/12 horas e acesso à água e ração padrão (Nuvital® Ltda., Curitiba, PR, Brasil) *ad libitum* até o momento do experimento. Todos os grupos foram pesados semanalmente para avaliação do peso corporal.

Os animais foram anestesiados com Halotano®, um anestésico sistêmico inalatório que possui ação anestésica e analgésica e promove rápido e reversível relaxamento muscular para coleta de sangue por punção cardíaca. Os animais foram eutanasiados pelo método de decapitação para coleta de fígado. Todos os procedimentos experimentais foram conduzidos segundo as Normas Internacionais de Pesquisa envolvendo Animais e o estudo foi aprovado pela Comissão de Ética no Uso Animal (CEUA) da UNIFESP sob o número 484411 (Anexo 1).

4.2 Grupos experimentais

Os animais foram separados aleatoriamente em quatro grupos, com 5 animais cada, descritos a seguir:

- Controle: animais submetidos a uma única injeção intraperitoneal de solução salina 0,9% e, após 15 dias, gavage diária com água filtrada (1 mL) durante 15 dias.

- Cádmio: animais submetidos a uma única injeção intraperitoneal de 1,2 mg/kg de cloreto de cádmio e, após 15 dias, gavage diária com água (1 mL) durante 15 dias.

- Cádmio + SU: animais submetidos a uma única injeção intraperitoneal de 1,2 mg/kg peso corporal de cloreto de cádmio e, após 15 dias, gavage diária de concentrado de suco de uva (0,8 mL) por 15 dias.
- Cádmio + SM: animais submetidos a uma única injeção intraperitoneal de 1,2 mg/kg peso corporal de cloreto de cádmio e, após 15 dias, gavage diária (1 mL) de suco de maçã por 15 dias.

4.3 Tratamentos

4.3.1 Cloreto de Cádmio

Os animais tratados com cádmio receberam uma única injeção intraperitoneal de cloreto de cádmio Nuclear® na dosagem de 1,2 mg/kg de peso corporal, de acordo com o protocolo padronizado por Predes et al. (2010).

4.3.2 Concentrado de Suco de Uva

O concentrado de suco de uva utilizado G8000® foi fornecido pela empresa Golden Sucos® (Farroupilha, RS, Brasil). A matéria-prima utilizada na elaboração do concentrado são uvas das variedades Bordô e Concord. Em estudo prévio do nosso grupo e pesquisa, Aguiar-Jr et al. (2011) realizou a caracterização quimica por espectrometria de massa e avaliou a capacidade antioxidante do concentrado de suco de uva utilizado G8000®, demonstrando que o produto possui uma atividade antioxidante de 27,03 g de Vitamina C eq/kg (equivalentes de Vitamina C/kg) e teor de fenólicos totais de 45,8 GAEg/kg (equivalentes de ácido gálico/kg). Ele contém dissacarídeos, ácido graxos (ácido palmítico e linoleico), ácidos orgânicos (ácidos caffeoiltartarico, fertárico e caffeoilquinico), antocianidinas (caempferol-galactosídeo, petunidina 3-O-acetilglucosídeo, peonidina-glucosídeo, peonidina 3-p-coumaroilglucosídeo malvidina-glucosídeo e malvidina 3-O-p-coumaroilglucosídeo), além de quercetina e resveratrol.

De acordo com *American Dietetic Association* há evidências de que o consumo diário de 250-500 mL de suco de uva ou vinho demonstram efeitos fisiológicos dos polifenólicos em humanos (Hasler et al. 2004). A dosagem de G8000® utilizada no presente estudo foi calculada com base no consumo humano que é de aproximadamente quatro copos contendo 200 mL de suco de uva natural (Gollucke et al., 2008). Desta forma, como o suco possui 45,8 g/kg de fenóis totais e a dose máxima efetiva de suco de uva (1000 mL) contém aproximadamente 2 g de polifenóis, a dose para ratos (pesando aproximadamente 300 g) equivalente à dos humanos é de aproximadamente 16 mg de polifenóis por dia. Considerando o metabolismo murino ser duas vezes mais acelerado que o ser humano, esse valor correspondeu a 355 mg de G8000®, administrado via oral (gavage) por meio de uma seringa para essa finalidade.

4.3.3 Concentrado do Suco de Maçã

O suco concentrado de maçã, produzido a partir do bagaço do fruto, foi também fornecido pela Golden Sucos® (Farroupilha, RS, Brasil). De acordo com análise prévia, o suco de maçã utilizado neste trabalho possui concentração de 4,37 g de fenóis totais e atividade antioxidante de 1,21 g de Vitamina C eq/kg e é composto por ácido caffeoilquinico, ácido heptacosanóico, 1-dodecanoil-glicero-3-fosfo-(10-snglicerol) e cianidina-3-O-glucosídeo (Ribeiro et al. 2014).

Segundo Kahle e colaboradores (2007) e van der Sluis et al. (2001), estima-se que a quantidade diária de ingestão de flavonóides estar-se-á entre 0,15 e 1 g de fenóis totais. A maçã possui, em média, 66,2 a 211,9 mg de polifenóis para cada 100 g de fruto, dependendo da variedade (Vrhovsek et al. 2004).

Da mesma forma, o cálculo para a dosagem a ser administrada de suco de maçã neste estudo levou em consideração as recomendações da *American Dietetic Association* utilizadas para o concentrado de suco de uva G8000®. Entretanto, seria necessário ofertar uma dose de, aproximadamente, 7 mL de suco de maçã para que os animais recebessem a mesma quantidade de polifenóis. Como a administração desta quantidade de suco seria inviável por gavage, decidiu-se administrar 1 mL do

concentrado do suco de maçã, que equivale ao volume máximo a ser administrado sem provocar qualquer desconforto ou malefício aos animais.

4.4 Análises

4.4.1 Análise de estresse oxidativo por imunoistoquímica

Completado os períodos experimentais estabelecidos, os animais foram anestesiados e eutanasiados por excesso de solução anestesica. Os fígados foram removidos e fixados em formol a 10% por 24 horas para posterior processamento histológico. Para análise de estresse oxidativo foram avaliados a expressão de biomarcadores para a detecção de 8-OHdG (8-hidroxi-20-guanosina) pela técnica de imunoistoquímica em tecido hepático. Para tanto, cortes seriados de 3 µm foram desparafinados em xilol, reidratados em etanol (99.5%) e pré-tratados com tampão de ácido-cítrico (10 nM, pH 6, 0,1 M ácido cítrico, Synth®, São Paulo, Brasil; 0,1 M citrato de sódio – Synth®, São Paulo, Brasil) em micro-ondas, por três ciclos de cinco minutos cada para recuperação antigênica. Em seguida, os cortes foram incubados com anti-8-OHdG (Santa Cruz Biotechnologies Inc.®, EUA) na diluição de 1:100, *overnight*, a 4ºC. Os espécimes foram então submetidos a duas lavagens com tampão fosfato de sódio (PBS) e os cortes foram incubados com o anticorpo secundário por 45 minutos, e em seguida, corado com DAB (3,3-diaminobenzidina, 0,05%) (DAKO North America Inc.®, Califórnia, EUA) e contra-corados com a hematoxilina de Harris (Sigma®, Missouri, EUA). Uma vez realizada a marcação, foram avaliados 1000 hepatócitos por lâmina em microscópio de luz (Nikon®, Model Eclipse E200, Japão), em aumento de 400x, por sistema de casualização sistemática.

4.4.2 Análise de mutagenicidade pelo Teste do Micronúcleo

O teste do micronúcleo em medula óssea foi realizado de acordo com o protocolo estabelecido por Ribeiro et al. (2008) no qual o osso fêmur foi coletado e mantido em solução de cloreto de sódio a 0.9%. A epífise proximal dos ossos foi

removida e 1 mL de soro fetal bovino (Cultilab®, Campinas, Brasil) foi injetado no canal medular. Foram confeccionados esfregaços em lâminas de vidro previamente limpas a partir da suspensão formada pela medula óssea e soro fetal bovino. Após as lâminas secarem em temperatura ambiente por 24 horas, as mesmas foram coradas em Giemsa (Merck®, Alemanha).

O teste do micronúcleo em fígado foi realizado em cortes parafinados seriados de 3 µm e corados por Schiff (Merck®, Alemanha) e contra-corados com Fast Green® (Sigma Aldrich®, EUA).

Para a análise de resultados, foram contabilizados 1000 eritrócitos policromáticos ou hepatócitos por animal, utilizando-se microscópio de luz no aumento de 100x.

4.4.3 Análise de genotoxicidade por meio do teste em células individualizadas em gel de agarose (teste do cometa) em sangue periférico e fígado

O protocolo utilizado para a avaliação de genotoxicidade seguiu as orientações propostas por Tice et al. (2000). Sangue periférico e hepatócitos provenientes de macerado do fígado em PBS foram transferidos para tubos individuais contendo 1 mL de solução gelada de tampão fosfato (PBS, livre de cálcio e magnésio, pH 7,3) e centrifugados por cinco minutos, a 1000 rpm, em temperatura ambiente. O sobrenadante foi removido e as células utilizadas para teste do cometa.

Um volume de 10 mL de células de sangue periférico ou do fígado foram adicionados a 120 µl de agarose a 0,5% de baixo ponto de fusão a 37°C e a solução depositada em uma lâmina pré-revestida com agarose 1,5% regular (Life Technologies®, Auckland, Nova Zelândia) e coberta com uma lamínula (procedimento realizado em duplicata). Após a solidificação da agarose em geladeira, a lamínula foi retirada e as lâminas foram então imersas em solução de lise contendo 2,5 M de cloreto de sódio (Merck®, Alemanha) com 100 mL de etileno-diamino-tetracético (EDTA, Merck®, Alemanha), 10 mM de tampão Tris-HCl (hidrocloreto hidroximetil-aminometano, pH 10, Sigma Aldrich ®, EUA), sarcosinato de sódio a 1% (Sigma®,

EUA), Triton X-100 a 1% (4-(1,1,3,3-Tetrametilbutil)fenil-polietileno glicol, Sigma®, EUA) e dimetilsulfóxido a 10% (DMSO, Merck®, Alemanha) por cerca de uma hora. Antes da eletroforese, as lâminas foram lavadas em PBS gelado por cinco minutos, deixadas em tampão alcalino (0,3 mM de hidróxido de sódio e 1 mM EDTA (pH>13, Merck®, Alemanha) por 20 minutos e a eletroforese foi realizada neste mesmo tampão por 20 minutos, a 25 V e 300 mA. Após a eletroforese, as lâminas foram neutralizadas em 0,4 M Tris-HCl (pH 7,5, Sigma Aldrich®, EUA) para então serem fixadas em etanol absoluto e armazenados em temperatura ambiente até o momento da análise. A fim de minimizar os danos do DNA oriundos da radiação ultravioleta do ambiente, todos os procedimentos foram feitos com iluminação reduzida.

Um total de 50 cometas por animal, foi capturado randomicamente (25 células de cada lâmina de cada animal) e examinados em microscópio de fluorescência (Olympus®, EUA) conectado a uma câmera e sistema de análise de imagem (Comet Assay II®, Perceptive Instruments®, Grã-Bretanha). Para a mensuração do dano ao DNA foram considerados os seguintes parâmetros: intensidade da cauda (porcentagem do DNA migrado) e momento da cauda (produto da extensão da cauda e a fração do DNA na cauda do cometa) (Tice et al. 2000).

4.4.4 Análise de danos oxidativos genômicos por meio do teste do cometa modificado (teste do desafio)

A fim de avaliar o efeito protetor dos sucos concentrado de uva G8000® e de maçã perante a genotoxicidade induzida pelo cádmio, foi realizado o teste do desafio com peróxido de hidrogênio (H_2O_2) ou 4-NqO em hepatócitos a partir da técnica do cometa modificada. Para tanto, após a maceração do fígado em PBS, conforme descrito no teste do cometa anteriormente, hepatócitos foram tratados com H_2O_2 (0,6 mM) por cinco minutos ou com 4-NqO na concentração de 10 μM por 15 minutos. Após este período, foi adicionado agarose às células, confeccionados esfregaços em lâminas previamente cobertas com uma camada de agarose e levadas à solução de lise para, posteriormente submetê-las à eletroforese de acordo com o procedimento já descrito acima para o teste do cometa convencional.

4.4.5 Análise de expressão gênica de enzimas antioxidantes através da técnica da Cadeia de Polimerase Reversa (PCR) em Tempo Real (qPCR)

Para a análise da expressão gênica por meio da técnica de PCR em tempo real (qPCR), foi feita a extração do RNA total do tecido congelado, em protocolo adaptado de Chomczynski e Sacchi (1987). O tecido foi homogeneizado em 1 mL de Trizol (Invitrogen®, California, USA) e, em seguida, foram adicionados clorofórmio, isopropanol e etanol 75%. O *pellet* formado foi ressuspenso em 40 µl de água tratada com DEPC (dietilpirocarbonato 0,1%; UltraPure®, Invitrogen®, California, USA). As amostras foram armazenadas em biofreezer a -80ºC. Para a quantificação da concentração de RNA e o grau de pureza da amostra foi utilizado o equipamento Nanodrop 2000c® (Thermo Scientific®, Canadá) cuja faixa de leitura para o RNA utilizada foi de 260/280 nm.

O RNA extraído foi então tratado com DNAse (desoxirribonuclease, Deoxyribonuclease Amp Grade I®, Invitrogen®, California, USA), conforme indicação do fabricante, a fim de eliminar restos de DNA na amostra. O volume final da reação foi de 10 µL.

Após a purificação do RNA, foi realizada a construção do cDNA (DNA complementar), por meio da reação de transcriptase reversa, utilizando-se o kit High-Capacity cDNA Reverser Transcription® (Applied Biosystems®, USA), conforme indicação do fabricante, com volume final de 20 µL.

A análise da expressão gênica utilizou *primers* previamente desenhados para os genes de interesse e para o controle endógeno gliceraldeído trifosfato desidrogenase (GAPDH). As sequências dos genes analisados encontram-se na Tabela 1.

A detecção da amplificação do cDNA da amostra foi realizada por uso de intercalante de DNA (Sybr Green® – Applied Biosystems®, USA). Para tanto, foi preparado um mix contendo Sybr Green® (Sybr Green Master Mix®, Applied Biosystems®, USA), sequências iniciadoras (*primers*) *sense* e *anti-sense*, amostra e água DEPC q.s.p. 20 µL (UltraPure®, Invitrogen®, Califórnia, USA). As amostras foram pipetadas em duplicatas na placa óptica e coberta com cover óptico e analisadas no

equipamento Real Time PCR 7500 Fast® (Applied Biosystems®, USA) seguindo-se o programa de ciclagens: pré-incubação -95ºC por 10 minutos, 40 ciclos de 15 segundos a 95ºC e a 60ºC por 1 minuto, finalizando com a curva de dissociação (curva de Melting): 95°C por 15 segundos, 60ºC por 1 minuto e 95ºC por 15 segundos. Os resultados foram obtidos por meio do método $2^{-\Delta\Delta Ct}$ tanto para o grupo controle como para os grupos tratados e normalizados em relação aos valores obtidos do gene endógeno.

Tabela 1: Sequência dos *primers* utilizados para os genes de interesse e controle endógeno

Gene	*sense*	*anti-sense*
SOD-Cu/Zn	5´-CCAGTGCAGGACCTCATTTT-3´	5´-CCTTTCCAGCAGTCACATTG-3´
SOD-Mn	5´-ACACATTAACGCGCAGATCA-3´	5 -AATATGTCCCCCACCATTGA-3´
Catalase	5'-AGCGGATTCCTGAGAGAGTG-3'	5'-GAGAATCGAACGGCAATAGG-3'
GAPDH	5´-CAACTCCCTCAAGATTGTCAGCAA-3´	5´-GGCATGGACTGTGGTCATGA-3´

4.5 Análise estatística

Os valores foram expressos como média ± desvio padrão. Os resultados obtidos nas análises imunoistoquímica, teste do micronúcleo, teste do cometa e qPCR foram comparados por Análise de Variância (ANOVA) uma via seguido pelo teste de comparações múltiplas de Tukey. Os resultados obtidos no teste do desafio foram comparados por ANOVA duas vias, seguido pelo teste de comparações múltiplas de Tukey. Os cálculos estatísticos foram realizados com o emprego do programa *Graph Pad Prism* versão 6.0. Foi adotado o nível de significância de 95% (p<0,05).

5 RESULTADOS

5.1 Artigo aceito para publicação no periódico *Critical Reviews in Food Science and Nutrition*

ARE FOOD COMPOUNDS ABLE TO MODULATE NOXIOUS ACTIVITIES INDUCED BY CADMIUM EXPOSURE?

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Abstract

Cadmium is one of the most toxic environmental and industrial pollutants and is able to induce severe injury because it is poorly excreted, accumulating in various organs. This common pollutant is responsible for serious damage in lung, brain, testis, kidney, liver, blood system and bone. Food compounds, such as flavonoids, represent the most abundant polyphenols in human dietary and comprise thousands of substances, which are freely available as high-dose dietary supplements. The mechanism of action of these ones consists in free radical scavenging and metal sequestration. The interaction of metal ions with flavonoids leads to chelation formation and the using of these natural compounds is better than the synthetic ones due to their lower toxic effects. The aim of this review is to describe the role of some food compounds, focusing flavonoids for modulating noxious biological activities induced by cadmium exposure.

Key words: flavonoids; cadmium; toxicity; nutrition

INTRODUCTION

In the last decades, chemoprevention has emerged as a novelty in controlling several chronic diseases, such as cardiovascular disease, renal dysfunction, lung, kidney and gastrointestinal damage, neuro-degeneration and cancer (Del Rio et al., 2013; Bishayee et al., 2011; Nordberg, 2009; Arora et al., 2008; Borges et al, 2008).

The understanding of the mechanisms of action involving chemoprotective compounds has been addressed by observing possible therapies against the toxic effects of heavy metals such as cadmium (Lawal & Ellis, 2011). Biologically, such metals participate in various signaling and metabolic pathways (Flora et al., 2013) and they are potentially toxic, mainly due to oxidative stress (Nwokocha et al., 2012).

Several metals and metalloids have been classified as being carcinogenic by the International Agency for Research on Cancer (Ziech et al., 2010; Mena et al., 2009). The potential of different metals on generating reactive oxygen species (ROS) is considered the main mechanism of metal-induced carcinogenesis since they are capable of changing the redox mechanism in the eukaryotic cells (Lee et al., 2012). Numerous studies have shown an increase in the incidence of cancer associated with chronic exposure to heavy metals (Arita & Costa, 2009).

Cadmium is one of the most toxic environmental and industrial pollutants and is able to induce severe injuries. It is poorly excreted and accumulates in various organs, being responsible for serious damages in lung, brain, testis, kidney, liver, blood system and bone (El-Refaiy & Eissa, 2013; Anetor, 2012). Diet is the main source of environmental exposure to cadmium in non-smokers; it is present in practically every food type (Engström et al., 2011; Satarug et al., 2010; Jarup & Akesson, 2009; Panjehpour & Bayesteh, 2008; Brzóska et al., 2003). According to Nasreddine & Parent-Massin (2002), two thirds of the cadmium exposure through the diet is attributed to contaminated vegetables and the one third to animal products. The World Health Organization states that the human daily intake of cadmium ranges from 40 mg in non-polluted regions to 200 mg for contaminated areas (Ivanova et al., 2013). Moreover, smoking rises cadmium exposure in active and passive smokers (Satarug, 2012).

Studies have demonstrated that cadmium is absorbed by the gastrointestinal tract and by pulmonary exposure, being carried via blood bonded to albumin (Nordberg, 2009; Sánchez-González et al., 2006). On the liver, this complex inhibits liver enzymes promoting increased lipid peroxidation, congestion, ischemia and hypoxia (Sánchez-González et al., 2006). Concomitantly, it induces the synthesis of metallothionein (MT), a chain of aminoacids with a binding site for metals. The cadmium-MT complex is filtered by the renal glomeruli and reabsorbed by the proximal tubules. There, digestive enzymes act on the protein, releasing the cadmium ions restarting the synthesis of new MT. When the levels of free cadmium exceed the production of MT, extensive damage in the kidney cell membranes occurs and, consequently, renal insufficiency takes place (Sanchez-Gonzalez et al., 2006. Zhao et al., 2010; Nordberg, 1984).

In the lungs, the absorption and accumulation of cadmium are responsible for the development of diseases such as emphysema, bronchitis and cancer (Panjehpour & Bayesteh, 2008). Zhao and colleagues (2010) argued that there is an increase of MT response in the lungs, possibly as a defense mechanism against the presence of cadmium in the tissue.

Regardless of the affected tissue, toxicity triggered by cadmium is initially characterized by oxidative damage (Eybl et al., 2006). Studies have suggested that the mechanism of acute toxicity by cadmium involves depletion of glutathione and proteins bound to a sulfhydryl group. The result is in an increase of reactive oxygen species, which, in turn, promotes lipid peroxidation, leading to DNA damages (El-Refaiy & Eissa, 2013; Liu et al., 2009).

In view of the extensive human exposure to cadmium (through environmental, occupational and food contamination, as well as smoking habits) studies demonstrating the effectiveness of food compounds in reducing or reversing the damage are welcome.

Numerous food compounds have been tested for this purpose. Flavonoids represent the most abundant polyphenols in human diet, comprising thousands of compounds (de Moura et al., 2013), which are also freely available as dietary supplements (Flora et al., 2013). Biologically, the mechanism of action attributed to

polyphenols is of free radical scavenging and metal sequestration (Flora et al., 2013; Fraga et al., 2010). The inactivation of free radicals occurs when an antioxidant reacts with the free radical and even when the reaction leads to the formation of another, less active, radical (Leopoldini et al., 2011).

In spite of the therapeutic interventions using potent chelating agents for cadmium intoxication-treatment, an effective chelation therapy has not yet been established (Flora & Pachauri, 2010). Nevertheless, great efforts have been made in order to test the use of antioxidants for treating genomic instability syndromes induced by cadmium (Anetor, 2012, (Ramesh & Satakopan, 2010). Therefore, it is believed that the use of natural antioxidant compounds is one way to attenuate the damage caused by cadmium intoxication (Renugadevi & Prabu, 2010a).

Regarding the chelation mechanism, chelating agents are able to remove toxic metals, forming a stable complex. The metal is then removed from the biological chelator and this new complex, non-toxic, can be transported across physiological barriers, facilitating cadmium excretion not only from the site of deposition, but also from the body (Flora et al., 2013).

Several natural compounds have been described in the literature due to chelating roles (Sears, 2013). Chelators are able of mobilize metals in the tissues and maintain the chelate moiety during circulation to the kidneys (for excretion in the urine) and to the liver (for excretion in the bile) (Sears, 2013).

The aim of this review is to describe the role of food components in modulating noxious activities induced by cadmium exposure.

Studies involving the use of food components and cadmium exposure

Since 1980, polyphenols have been tested as chelator agents. The interaction of metal ions with flavonoids forms complexes with the advantage of showing lower toxic effects in comparison with synthetic chelators (Smith, 2013; Symonowicz & Kolanek, 2011; Malešev & Kunti 2007). Recently Gollücke et al. (2013) revised recent uses of polyphenols against diseases and discussed several possible mechanisms.

Quercetin, a biologically active and common dietary flavonoid present in fruits and vegetables, is known by its antioxidant activity. It has been reported that the substance is able to prevent oxidative injury and cell death by chelating metal ions, scavenging oxygen radicals, and protecting against lipid peroxidation (Unsal et al., 2014; Bu et al., 2011; Malešev & Kunti, 2007). Unsal and colleagues (2014) evaluated the neuroprotective effect of quercetin against cadmium exposure. The authors injected cadmium chloride (2 ml/kg/day for 30 days) in Sprague-Dawley rats, and the quercetin-treated group received 15mg/kg once a day intraperitoneally (ip) starting 2 days prior to cadmium injection. After the experimental period enzymatic antioxidants (superoxide dismutase, glutathione peroxidase and catalase) and lipid peroxidation levels were evaluated. The authors concluded that quercetin was effective in combating cadmium-induced neurotoxicity.

Wang and colleagues (2013) studied quercetin effects on cadmium-induced cytotoxicity in proximal tubular cells (1µg/mL quercetin; 2.5 or 5µmol/L cadmium) and showed that this compound had a protective effect in the cell against cadmium damages, inhibiting apoptosis, attenuating lipid peroxidation and renewing mitochondrial function by elevation of antioxidant levels. Bu et al. (2011) also used quercetin in treating germ cells after cadmium intoxication, with 4 mg/kg of body weight of cadmium to mice, daily, during 2 weeks. Animals exposed to cadmium and treated with quercetin had the structure of the seminiferous epithelium and the antioxidant status restored to normal. These effects were confirmed by evaluating glutathione and superoxide dismutase status, as well as verifying the suppression of apoptosis of germ cells.

Renugadevi and Prabu (2010b) demonstrated that quercetin provided protective effect against oxidative damage induced by cadmium in the kidney tissue of rats (5mg/kg $CdCl_2$, 50mg/kg quercetin, for 4 weeks). The substance was able to re-establish the levels of the enzymatic antioxidants catalase, superoxide dismutase, glutathione peroxidase, S-transferase and reductase, and of non-enzymatic antioxidants (vitamins C and E and reduced glutathione) in the kidney, diminishing the lipid peroxidation and some other biochemical parameters, such as urea, uric acid and creatinine. Furthermore, quercetin also ameliorated the tubular necrosis, tubular degeneration, desquamation and thickening of basement membrane. In the heart, pre-treatment with quercetin prevented oxidative impairment in rats exposed to cadmium

reducing the activities of the cardiac enzymes (creatinine kinase, lactate dehydrogenase, alkaline phosphatase, aspartate transaminase and alanine transaminase) restoring to normal levels. The authors attribute the positive results to the anti-lipoperoxidative, anti-oxidant and membrane stabilizing properties of quercetin. Moreover, this food compound normalized serum lipids inhibiting the accumulation of cholesterol and consequently hypercholesterolemia and atherosclerosis. Histologically, quercetin was capable of preventing miofibrile damage induced by cadmium. Taken together, quercetin supplements protected cardiac tissue against heavy metals (Prabu et al., 2011).

Regardless of all the protective effects demonstrated by quercetin in cadmium-induced damage, discussed above, it was not able to protect hepatotoxicity according to Vicente-Sanchez and colleagues (2008). The authors showed that quercetin was not a metal chelator, although it prevented oxidative stress, increased metallothionenin and eNOS expression. They also suggested that lipid peroxidation may contribute to the liver damage produced by acute cadmium administration, but that is not the major toxic mechanism.

Naringenin, a flavonone found in citrus and grape fruits, was employed in cadmium-induced hepatotoxicity (5mg/kg $CdCl_2$, 50mg/kg naringenin, for 4 weeks). The results showed that naringenin significantly reversed the activities of serum hepatic marker enzymes to their near-normal levels, reduced lipid peroxidation, restored the levels of antioxidant defense in the liver and preserved the normal histological architecture of the tissue (Renugadevi & Prabu, 2010a). The association of naringenin with vitamins C and E accelerated the detoxication of cadmium in the liver tissue, inhibiting oxidative stress, improving antioxidant status and reducing histopathological changes (Prabu et al., 2011).

Many researchers indicate that honey has functional properties in human health which is mainly credited to the presence of flavonoid compounds (Abdelaziz et al., 2013). Honey was evaluated against cadmium exposure using rabbit's haematological parameters (Abdelaziz et al, 2013). Animals received 3mg/kg of cadmium (ip) and cadmium chloride in tap water (100mg/L). The results showed that the group receiving cadmium had an increase in glucose, total cholesterol and triglycerides levels at the end of the experiments, when compared to the control group. When the animals were

treated with honey, these parameters were attenuated when compared to the intoxicated animals but still higher than the control group. Serum transaminases (aspartate aminotransferase, AST, and alanine aminotransferase, ALT), bilirubin and serum alkaline phosphatase (ALP) were also evaluated and they were ameliorated after the honey intake, as well as urea, uric acid and creatinine levels. The authors concluded that honey was effective in providing recoveries in the altered blood parameters.

Zingiber officinale is a herb used for culinary purposes which is also used therapeutically. It is known for anti-inflammatory and antioxidant potentials, including against metal toxicity, as a chelator agent. With regards to this property, Nwokocha et al. (2012) analyzed the hepatoprotection of *Zingiber officinale* to against heavy metals accumulation. This herb was mixed in rat chow (7% w/w) and fed to the animals exposed to cadmium (200 ppm in tap water). The results showed that *Zingiber officinale* affected the bioavailability, kinetics and assimilation of heavy metals as well as its uptake and excretion in a time-dependent manner, mainly acting as a hepatic protection against cadmium.

The most widely consumed beverage in the world, black tea, is rich in catequins that, when oxidized and polymerized, result in aflavin. This compound was tested for its protective effects against damages caused by cadmium in spermatogenesis (Wang et al., 2012). The authors applied 0.4 mg/kg of cadmium, once a day, during five weeks and treated the rats with 50, 100 or 200mg/kg of aflavin for the same period. The results showed that aflavin presented a dose-dependent response in most of the parameters, ameliorating sperm concentration, mobility and malformation, DNA damage of testicular cells, antioxidants enzyme levels (glutathiones and superoxide dismutase) and lipid peroxidation. The aflavins also had chelant action, diminishing cadmium-concentration in the liver, testis and blood level, while cadmium levels increased in urine and feces. In a similar manner, Wang and colleagues concluded that flavins were beneficial in the treatment of cadmium-induced testicular toxicity. When long-term grape juice concentrate consumption rich in polyphenols was tested, the results revealed that this compound was able to protect the reproductive parameters of cadmium-exposed male rats (Pires et al., 2013).

Curcumin and resveratrol were also studied by Ebyl et al. (2006) in a 3 day oral pre-treatment on cadmium-induced oxidative damage and cadmium distribution. According to the authors, the interaction between cadmium and curcumin reduced heavy metal load in the body and, resveratrol was able to inhibit lipid peroxidation. The data showed that both compounds completely prevented lipid peroxidation in the liver, but were not able to prevent glutathione depletion. Both curcumin and resveratrol prevented cadmium-induced inhibition of glutathione peroxidase, while resveratrol alone was effective against catalase activity inhibition. Conversely, none of the xenobiotics was able to reduce cadmium concentrations in biological tissues.

In 2002, Casalino and colleagues reported the effects of hydroxytyrosol (2-3,4-dihydroxyphenyletanol, DPE), a compound present in extra-virgin olive oil, combined with manganese (Mn^{2+}) in the liver of rats exposed to cadmium. The results showed that twenty four hours following cadmium intoxication, liver glutathione levels increased 30.4% in non-treated animals and similar result was observed with administration of a low concentration of Mn^{2+} (32.9%). The treatment of cadmium-exposed rats with Mn^{2+} alone failed to restore the liver activities of CuZn-SOD, Mn-SOD and catalase. Animals exposed to cadmium (2.5mg/kg, ip) and treated with DPE (9mg/kg, ip) and Mn^{2+}(2mg/kg, ip) were able to maintain the lipid peroxidation The authors concluded that the exposure to cadmium stimulated endogenous defense in liver cells and that DPE was able to modulate this outcome.

Two widely accepted foodstuffs are recognized for their culinary and medicinal properties: onion and garlic (Suru, 2008). *Allium cepa* (onion) and *Allium sativum* (garlic) are rich in antioxidant compounds and their protection potential against cadmium exposure has been documented (Ige et al., 2009). Ola-Mudathir and colleagues (2008) showed that onion and garlic extracts partially protects the testis and spermatozoa against cadmium toxicity by reduction in the level of lipid peroxidation as well as enhanced levels of glutathione, superoxide dismutase and catalase in the testis. The impairment in sperm characteristics was markedly restored with a corresponding increase in the weight of the testis. *Allium cepa* extract diminished superoxide dismutase and catalase enzymes as well as lipid peroxidation level. The extract was also able to decrease cadmium levels in urine of treated rats, preventing renal tubular damage. (Ige et al., 2011; Ige et al., 2009). Suru (2008) also obtained positive results for renal damage induced by cadmium and treated with *Allium cepa* or

Allium sativum. Onion and garlic had protective effects on cadmium induced oxidative stress by reduction of lipid peroxidation and by sparing the depletion of endogenous glutathione, superoxide dismutase and catalase. Onion and garlic intake offered a consistent protection against cadmium induced oxidative damage in the kidney.

Concluding remarks

In this article, we have showed recent studies focusing the modulatory activities exerted by food compounds against cadmium exposure using experimental test systems. Although these data have revealed important mechanisms of action much remains to be examined. More adequately powered, randomised, placebo controlled human studies are needed better understanding the role of these food compounds on human health. Therefore, this area warrants intensive investigation as a new way of knowledge, which would apply foods as promising therapeutic agent against human diseases.

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Conflict of Interest

None declared.

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ANTIMUTAGENIC AND ANTIGENOTOXIC POTENTIAL OF GRAPE JUICE CONCENTRATE IN BLOOD AND LIVER OF RATS EXPOSED TO CADMIUM

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ABSTRACT

The aim of this study was to evaluate the antimutagenic and antigenotoxic potential of grape juice concentrate in rodent organs exposed to cadmium chloride intox- ication. A total of 15 Wistar rats were distributed into three groups (n=5), as follows: control group (CTRL; nontreated group), cadmium group (Cd), and cadmium-grape juice group (Cd+GJ). Exposed animals received intraperitoneal injection of cadmium chloride (1.2 mg/kg body weight) diluted in water and, after 15 days, Cd+GJ group received grape juice con- centrate for 15 days, by gavage (0.8 mL, 1.18 mg of polyphenols kg^{-1} day^{-1}). Grape juice concentrate was able to decrease genotoxic effects induced by cadmium in peripheral blood and liver cells as depicted by single cell gel (comet) and micronucleus assays. A decrease for anti-8-hydroxy-20-deoxyguanosine (8OHdG) expression in hepatocytes of ani- mals exposed to cadmium and treated with grape juice con- centrate was also detected. Higher CuZn-SOD activity was observed in liver cells of the Cd+GJ group. No remarkable differences were seen regarding Mn-SOD activity among groups. Taken together, our results demonstrate that grape juice concentrate was able to exert antimutagenic and antigenotoxic activities in blood and liver cells of rats exposed to cadmium.

Keywords: Grape juice. Cadmium. Genotoxicity. Mutagenicity. Oxidative stress. Rat.

INTRODUCTION

Cadmium is a relevant environmental pollutant, present in contaminated soil, water, air, foodstuffs, and in the smoke released by cigarettes (Prozialeck et al. 2009; Edwards et al. 2013). It is highly toxic, accumulating in various human tissues (Akerstrom et al. 2013) and, for that reason, being responsible for several pathologies such as renal dysfunction, skeletal disorders, cardiovascular diseases, lung, kidney, and gastrointestinal damage, as well as liver and salivary glands malfunction (Arora et al. 2008; Borges et al. 2008; Nordberg 2009). Diet is the main source of cadmium exposure in non- smokers, and it is present in practically every sort of food (Bzróska et al. 2003; Panjehpour and Bayesteh 2008; Järup and Åkesson 2009; Satarug et al. 2010; Engström et al. 2011). Particularly in developing countries, humans are at high risk of cadmium exposure (Filipič 2012).

Studies have suggested that the mechanism of cadmium toxicity involves depletion of glutathione and proteins bound to the sulfhydryl group, resulting in an increase of reactive oxygen species (ROS) which, in turn, promote lipid peroxi- dation resulting in damage to DNA (Liu et al. 2009). Herein, great efforts have been made in attempt to find safe and potent antioxidants from vegetables and fruits (Ramesh and Satakopan 2010). Hodkova et al. (2008) and Eybl et al. (2006) observed a decrease in lipid peroxidation and an improvement in adverse effects caused by cadmium toxicity using curcumin and melatonin, respectively. Eybl et al. (2006) also assessed the efficacy of resveratrol to reverse the inhibition of catalase activity triggered by the action of cadmium.

Resveratrol is the main polyphenolic compound synthesized by grapevine leaves in response to fungal infection or exposure to ultraviolet light (Frémont 2000). In the body, it acts as an antioxidant, reducing the formation of ROS, modulating the metabolism of lipids and lipoproteins, and inhibiting platelet aggregation. Resveratrol also shows anti-inflammatory effects by inhibiting inflammatory markers expression and antitumor activity through inhibition of gene expression of nuclear factor κB (NF-κB), as well as by reducing stimulation of cell proliferation and apoptosis in tumor cells

(Frémont 2000; Benitez et al. 2009; Colin et al. 2009; Luna et al. 2009). In a review study, Gollücke (2010) describes the improvement of lipoprotein metabolism, oxidative stress, and inflammatory markers in addition to the inhibition of platelet aggregation as some of the beneficial effects of polyphenols present in grapes. According to Rho and Kim (2006), products derived from grapes such as grape juice or pomace, are able to decrease lipid peroxidation and DNA damage, significantly delaying the onset of degenerative diseases.

Considering the forceful pieces of evidence on the beneficial effect of grapes against physiologic disorders, the purpose of this study was to evaluate the antimutagenic and antigenotoxic potential of grape juice in multiple rodent organs subjected to intoxication by cadmium chloride.

MATERIALS AND METHODS

Animals and experimental design

All experimental protocols involving animals are conformed to procedures described in the Principles for the Use of Laboratory Animals Guidelines. The study was approved by the Animal Ethics Committee of the Federal University of São Paulo, UNIFESP, SP, Brazil (protocol no. 484411).

A total of 15 Wistar rats (8 weeks old, weighing 250 g on average) were obtained from Development Center of Experimental Models for Medicine and Biology (CEDEME), Federal University of São Paulo, SP, Brazil. They were maintained under controlled conditions of temperature (23±1 °C), light–dark periods of 12 h, and free access to water and commercial diet (Nuvital™, Brazil). The rats were distributed in three groups (n=5), as follows: control group (CTRL; nontreated group), cadmium group (Cd), and cadmium-grape juice group (Cd+GJ).

CTRL group received a single intraperitoneal (ip) water injection while the groups Cd and Cd+GJ received a single ip injection of cadmium chloride (1.2 mg/kg body weight) diluted in water and, after 15 days, Cd+GJ group received grape juice

concentrate G8000™ (Golden Sucos™, Farroupilha, Brasil) for 15 days, by gavage (0.8 mL, containing 1.18 mg of polyphenols kg−1 body weight day−1). CTRL and Cd groups were treated with drinking water by gavage during the same experimental period. The grape juice group was not included into this experimental design because our research group has evaluated the protective potential of grape juice under different paradigms and the results have revealed that animals treated with grape juice only did not change such biological parameters investigated (Pires et al. 2013; Paiotti et al. 2013; de Jesus et al. 2014).

All animals were checked daily for behavior, and general health conditions and body mass was recorded weekly (data not shown). At the end of the experimental period, all animals were anesthetized with inhalational anesthetic halothane (Tanohalo™, Cristália™, SP, Brazil) and euthanatized for tissue collection.

The dose of grape juice concentrate was calculated on the basis of amount of polyphenols to be equivalent to four glasses (200 mL, each) of natural grape juice and adjusted to the animal metabolism (twice as fast as humans) (Gollücke et al. 2008). The individual dose of grape juice concentrate was also adjusted to animal weight during the experiment, diluting in drinking water in order to achieve this purpose. According to the American Dietetic Association, human consumption of approximately 200–500 mL presents moderate to strong evidence of a positive physiological effect (Hasler et al. 2009).

The chemical characterization of grape juice concentrate was performed in a previous study conducted by our research group (Aguiar et al. 2011). The following compounds were identified: dimethoxyflavylium, disaccharide, fatty acids (palmitic and linoleic acids), and flavonoids (kaempferol-galactoside, peonidinglucoside, malvidin-glucoside, petunidin 3-O-acetylglucoside, peonidin 3-p-coumaroylglucoside, and malvidin 3-O-p-coumaroylglucoside). Caffeoyltartaric, fertaric, and caffeoylquinic acids appeared as the main organic acids identified. Quercetin appeared as a single molecule and resveratrol was also identified in relatively significant abundance.

Micronucleus test

The micronucleus test was performed in bone marrow and liver tissue. The bone marrow micronucleus test was performed according to Ribeiro et al. (2008). For that, femoral bones were collected and stored in 0.9 % sodium chloride. The proximal epiphyses of the bones were removed and 1 mL of fetal bovine serum (FBS; Cultilab™, Campinas, Brazil) was injected into the medullar canal. A smear on glass slides was performed with the suspension formed by the bone marrow and FBS. After drying the slides, they were stained with Giemsa (Merck™, Darmstadt, Germany). For liver micronucleus test, paraffin sections (3 μm) were stained by Feulgen and counterstained with Fast Green (Sigma Aldrich™, USA). A total of 1,000 polychromatic erythrocytes or hepatocytes were analyzed per animal. Slides were scored blindly using a light microscope with a ×100 immersion objective.

Single cell gel (comet) assay

The protocol used for peripheral blood and liver cells followed the guidelines outlined by Tice et al. (2000). Peripheral blood was collected by cardiac puncture and liver cells by liver tissue maceration with PBS. Cells were transferred to individual plastic tubes, containing 1 mL of cold phosphate buffer solution (PBS; Ca+2, andMg+2 ions free, pH 7.3), and centrifuged for 5 min, 1,000 rpm, at room temperature. The supernatant was removed and the cell suspensions (~10 μL) were used for single cell gel (comet) assay. A volume of 10 μL of cellular suspension was added to 120 μL of 0.5 % low melting point agarose at 37 °C, layered onto a pre-coated slide with 1.5 % regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and the slides immersed in lysis solution (2.5M NaCl, 100 mM EDTA—Merck™, Darmstadt, Germany; 10 mM Tris–HCl buffer, pH 10—Sigma Aldrich™, St Louis, MO, EUA; 1 % sodium sarcosinate—Sigma™, St Louis, MO, EUA; with 1 % Triton X-100—Sigma™, St Louis, MO, EUA; 10 % dimethyl sulphoxide—Merck™, Darmstadt, Germany) for about 1 h. Afterwards, the slides were washed in ice-cold PBS (Ca+2 and Mg+2 ions free, pH 7.3) for 5 min, left in electrophoresis buffer (0.3 mM NaOH and 1 mM EDTA (Merck™, Darmstadt, Germany, pH >13) for DNA unwinding during 20 min and electrophoresed in the same

buffer for 20 min at 25 V (0.86 V/cm) and 300 mA. Following electrophoresis, slides were neutralized in 0.4 M Tris–HCl (pH 7.5; Sigma Aldrich™, St Louis, MO, EUA), fixed in absolute ethanol and stored at room temperature until analysis in a fluorescence microscope at ×400 magnification. All steps were performed under reduced light.

A total of 50 randomly captured comets per animal (25 cells from each slide) were examined blindly by one expert observer at ×400 magnification using a fluorescent microscope (Olympus™, Orangeburg, NY, USA). The microscope was connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments™, Suffolk, Haverhill, UK) calibrated previously according to the manufacturer's instructions. To measure DNA damage, two image analysis system parameters were considered: tail intensity (% migrated DNA) and tail moment (the product of the tail length and the fraction of DNA in the comet tail) (Tice et al. 2000). Since none of the groups showed significant differences between these parameters, we chose tail moment for the presentation of the results.

Challenge assay

Liver cells were obtained from liver maceration and divided into two aliquots of 20 µL each to study DNA damage due to mutagen sensitivity. For this purpose, the first aliquot was treated with 4-nitroquinoline-1-oxide (4NqO; 0.1 µM) for 15 min, and the second aliquot was treated with hydrogen peroxide (H_2O_2; 0.6 mM) for 5 min. Then, agarose was added and smears were made and left in lysis solution (2.5 M NaCl, 100 mM EDTA—Merck™, Darmstadt, Germany; 10 mM Tris–HCl buffer, pH 10—Sigma Aldrich™, St Louis, MO, EUA; 1 % sodium sarcosinate—Sigma™, St Louis, MO, EUA; with 1 % Triton X-100—Sigma™, St Louis, MO, EUA; 10 % dimethyl sulphoxide—Merck™, Darmstadt, Germany) for about 1 h. All the slides prepared were submerged into alkaline buffer (pH 10) for 20 min and thereafter electrophoresis was conducted for another 20 min, at 300 mA and 25 V. After that, the slides were neutralized in Tris–HCl solution (Sigma Aldrich™, St Louis, MO, EUA) and fixed at ethanol and stored at room temperature until analysis in a fluorescence microscope at ×400 magnification. The analysis was the same as described to the comet assay.

Imunohistochemistry

Liver serial sections of 4 μm were deparafinized in xylene and rehydrated in graded ethanol (99.5 %), then pretreated in a microwave with 10 mM citric acid buffer (pH 6, 0.1 M citric acid - Synth™, São Paulo, Brazil; 0.1 M sodium citrate - Synth™, São Paulo, Brazil) for three cycles of 5 min each for antigen retrieval. They were pre-incubated with 0.3 % hydrogen peroxide for inactivation of endogenous peroxidase and then blocked with 5 % normal goat serum for 30 min. The specimens were then incubated with anti-8-hydroxy-20-deoxyguanosine (8OHdG; Santa Cruz Biotechnologies Inc™, MO, USA) at 1:100 dilution overnight at 4 °C. This was followed by two washes in PBS and further incubation with a biotinylated secondary antibody, diluted 1:100 in PBS for 1 h. The sectionswere washed twice with PBS followed by the application of preformed avidin biotin complex (Vector Technologies™, USA) for 45 min. The bound complexes were visualized by the application of a 0.05 % solution of 3,3-diaminobenzidine (Sigma™, St Louis, MO, EUA) and counterstained with hematoxylin (Sigma™, St Louis, MO, EUA). Sections stained using immunohistochemistry were analyzed for the percentages of immunopositive cells in liver. A total of 1,000 hepatocytes were evaluated in 3 to 5 fields at ×400 magnification. These values were used as labeling indices.

Real Time PCR

Liver tissue at −86 °C was homogenized, and total RNA was isolated using cold Trizol Reagent (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was determined using a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies™, Wilmington, DE). RNA samples were treated with DNAse (DNase Amplification Grade™, Applied Biosystems™, Foster City, CA, USA) to avoid contamination with genomic DNA.

cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed in a 7500 Fast Real- Time PCR System (Applied Biosystems™, Foster City, CA, USA) using the Power SYBR™Green Kit (PCR Master Mix 2×, Applied Biosystems™, Foster City, CA, USA).

Primers for the specific amplification of each cDNA were designed using the Primer Express software (Applied Biosystems™, Foster City, CA, USA), considering established criteria, such as product length, optimal PCR annealing temperature, and the likelihood of primer self-annealing. The primers sequence are: GAPDH: sense 5′-CAA CTC CCT CAA GAT TGT CAG CAA-3′ and anti-sense 5′-GGC ATG GAC TGT GGT CAT GA-3′; SOD-Cu/Zn: sense 5′-CCA GTG CAG GAC CTC ATT TT-3′ and anti-sense 5′-CCT TTC CAG CAG TCA CAT TG-3′; SOD-Mn: sense 5′-AAC ATT AAC GCG CAG ATC A-3′ and anti-sense 5′-AATATG TCC CCC ACC ATT GA-3′.

PCR reactions were performed in duplicate containing 20 µL final volume using 2.0 µL of a 1:5 (v/v) dilution of cDNA, 2.0 µL of primer mix (forward and reverse), 10.0 µL of Power SYBR™ Green (PCR Master Mix 2×, Applied Biosystems™, Foster City, CA, USA), and DPEC water (Ultrapure™ DEPC Treated Water, Invitrogen™, Carlsbad, CA, USA). The reactions were performed in MicroAmp™ 96-well plates (Applied Biosystems™, Foster City, CA, USA) covered with optical adhesive (Applied Biosystems™, Foster City, CA, USA). Samples were submitted to 40 cycles of 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. An amplification efficiency curve using different cDNA dilutions was also performed for each gene tested.

To normalize the data for the control and experimental groups, arbitrary units were calculated as: arbitrary unit=2-ΔΔCT, and ΔΔCT=sample ΔCT−control ΔCT, where CT is the threshold cycle.

Statistical analysis

All data were expressed as mean±standard deviation (SD), and compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post hoc for micronucleus test, Single Cell Gel (Comet Assay), Immunohistochemistry and Real-Time PCR. The data obtained in Challenge Assay was analyzed by two-way ANOVA, followed by Tukey's multiple comparisons post hoc analysis. Statistical analysis was performed using GraphPad Prism™ 6.0 program $p<0.05$ was considered to be significant.

Results

Micronucleus test

Micronucleus test results in bone marrow and liver are shown at Figs. 1 and 2, respectively. Both results showed a significant increase in the number of micronucleus in cadmiumintoxicated animals. Comparing Cd and Cd+GJ groups, significant decrease in the frequency of micronucleated erythrocytes of animals treated with G8000™ was verified ($p<0.05$).

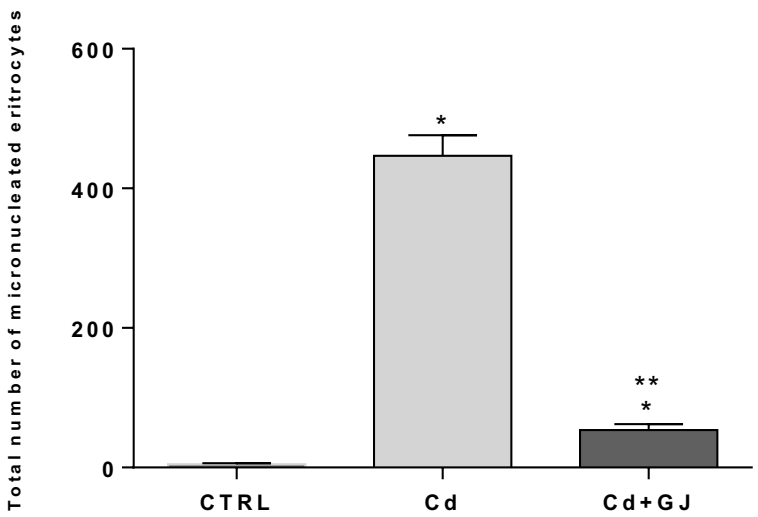

Fig. 1: Total number ofmicronucleated erythrocytes-bone marrow of rats exposed to cadmium and treated with grape juice concentrate G8000™. ANOVA followed Tukey's multiple comparisons post hoc. *$p<0.05$ when compared with group (CTRL); **$p<0.05$ when compared with cadmium group (Cd). Cd+GJ cadmium and grape juice group

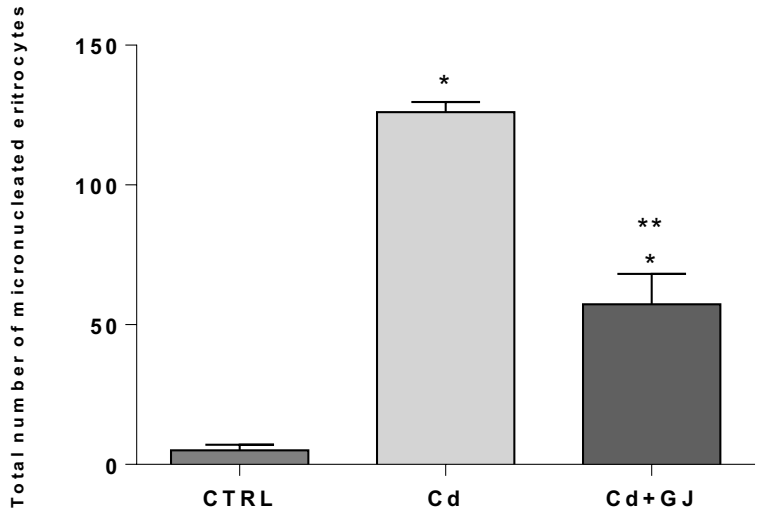

Fig. 2: Total number of micronucleated hepatocytes of rats exposed to cadmium and treated with grape juice concentrate G8000™. ANOVA followed Tukey's multiple comparisons post hoc. *$p<0.05$ when compared with group (CTRL); **$p<0.05$ when compared with cadmium group (Cd). Cd+GJ cadmium and grape juice group.

Comet assay and challenge assay

The results from single cell gel (Comet) assay are shown in Table 1. Peripheral blood data showed an increase in the tail moment measured for Cd group when compared with CTRL, whereas the group treated with grape juice concentrate G8000™ (Cd+GJ) showed a significant reduction in DNA damage. Similar results were observed in liver: Cd group showed significantly higher DNA damaging when compared with the CTRL group. Significant lower genetic damage in animals receiving injection of cadmium and treated with grape juice concentrate G8000™ (Cd+GJ) was noticed.

In this study, we were able to evaluate mutagen sensitivity induced 4NqO and H_2O_2 in rats exposed to cadmium and grape juice concentrate G8000™. Our results demonstrated that grape juice concentrate G8000™ was able to decrease the alkylation induced by 4NqO. In a similar manner, grape juice concentrate G8000™ was able to protect liver cells against the oxidative stress by H_2O_2. Such findings are demonstrated in Fig. 3.

Table 1: DNA damage in multiple in peripheral blood and liver of rats exposed to cadmium and treated with grape juice concentrate G8000™; by Comet assay

	Tail Moment		
	CTRL	**Cd**	**Cd+GJ**
Peripheral Blood	0.85 ± 0.25	2.83 ± 0.35 *	0.5 ± 0.08 **
LIver	0.8 ± 0.37	3.6 ± 1.07 *	1.13 ± 0.28 **

Two-way ANOVA followed Tukey's multiple comparisons post test. Cd+GJ: cadmium and grape juice group. * $p<0.05$ when compared with group (CTRL); ** $p<0.05$ when compared with cadmium group (Cd)

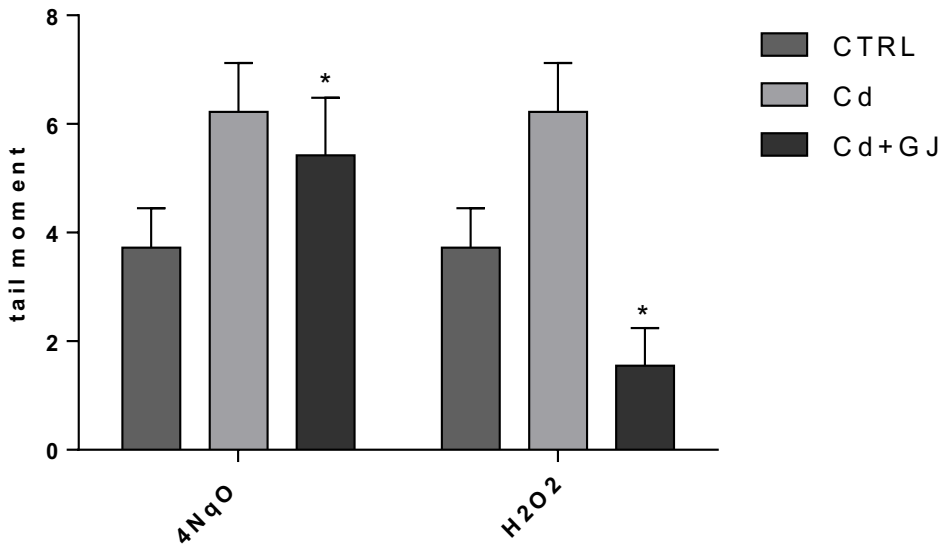

Fig. 3: Challenge assay: DNA damage in hepatocytes of rats exposed to cadmium and treated with grape juice concentrate G8000™and exposed to 4NqO and H_2O_2. Two-way ANOVA followed Tukey's multiple comparisons post hoc. *$p<0.05$ when compared with cadmium group (Cd); CTRL control group, Cd+GJ cadmium and grape juice.

Immunohistochemistry

The immunohistochemistry analysis showed a decrease in 8OHdG imunoexpression in the hepatocytes of animals exposed to cadmium and treated with grape juice concentrate G8000™ (Cd+GJ). These results can be better visualized in Figs. 4 and 5. Grape juice concentrate G8000™ was able to reduce 8OHdG levels when compared with Cd group, with statistical relevance between the groups ($p<0.05$).

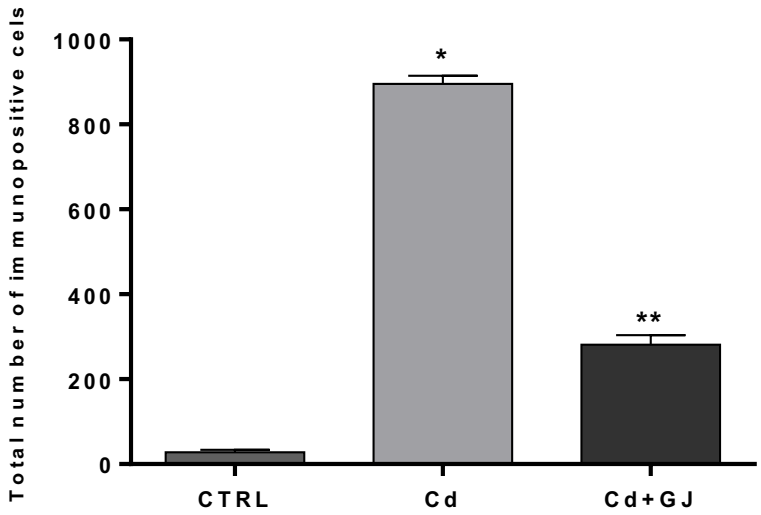

Fig. 4: Total number of immunopositive cells for 8OHdG. Two-way ANOVA followed Tukey's multiple comparisons post hoc. *$p<0.05$ when compared with group (CTRL); **$p<0.05$ when compared with cadmium group (Cd). Cd+GJ cadmium and grape juice group.

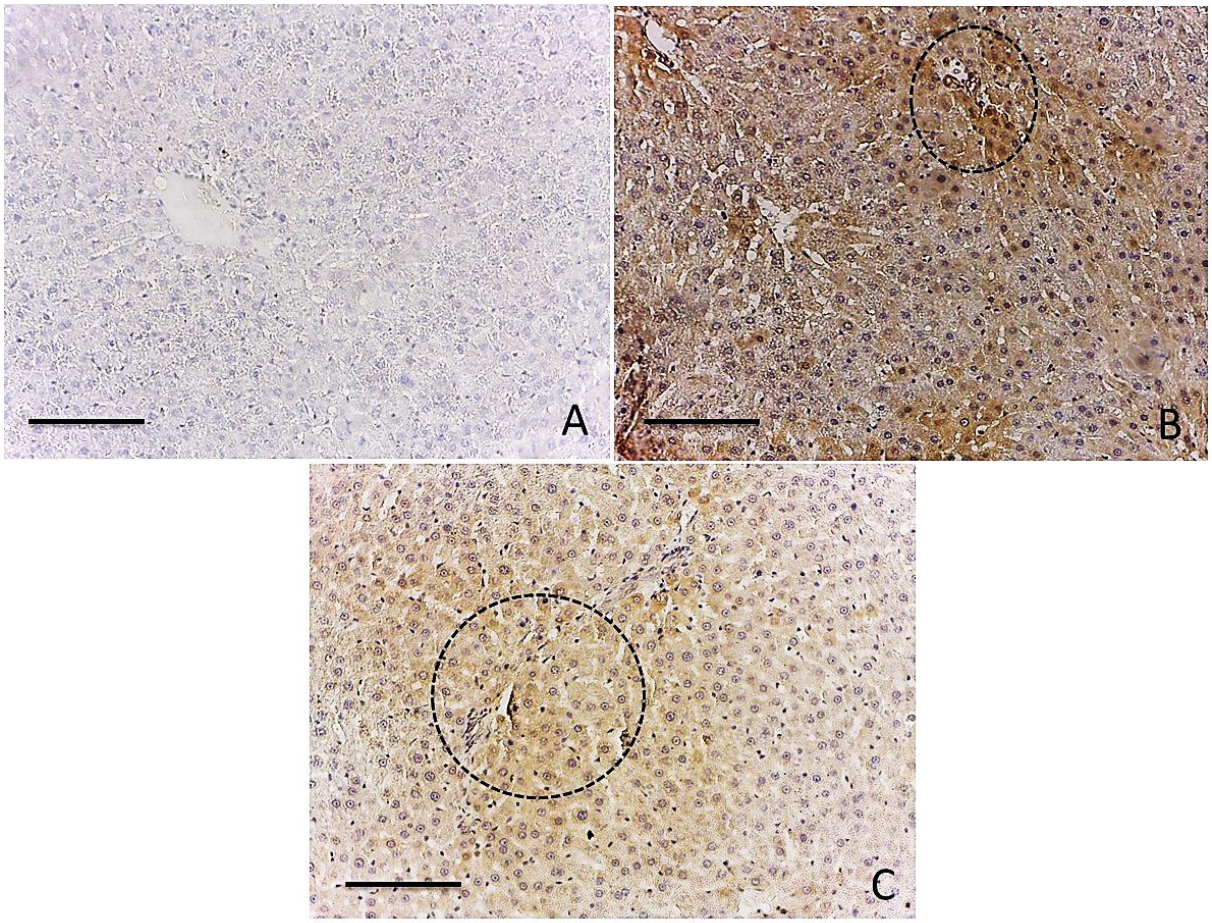

Fig. 5: Immunohistochemistry for 8OHdG: **a)** control group (CTRL); **b)** cadmiumgroup (Cd); **c)** grape juice concentrate G8000™-treated rats exposed to cadmium. Circle indicates positive expression in liver cells. Bar=75 µm.

Real-Time PCR

Antioxidant enzymes copper–zinc superoxide dismutase (CuZn-SOD) and manganese superoxide dismutase (Mn-SOD) were analyzed by real-time polymerase chain reaction (qPCR). The results showed an increase at CuZn-SOD expression in the Cd+GJ group when compared with Cd group (Fig. 6). Mn-SOD expression did not show remarkable differences among groups. Such findings are demonstrated in Figs. 6 and 7.

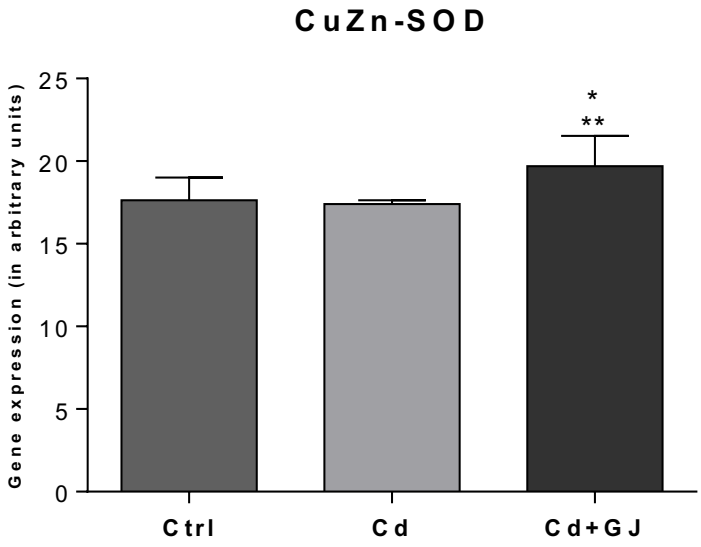

Fig. 6: Effects of grape juice concentrate G8000™ on the expression of Cu/Zn-SOD in liver of rats exposed to cadmium and treated with grape juice concentrate G8000™ using real-time PCR. ANOVA followed Tukey's multiple comparisons test. *p<0.05when compared with group (CTRL); **p<0.05 when compared with cadmium group (Cd). Cd+GJ cadmium and grape juice group.

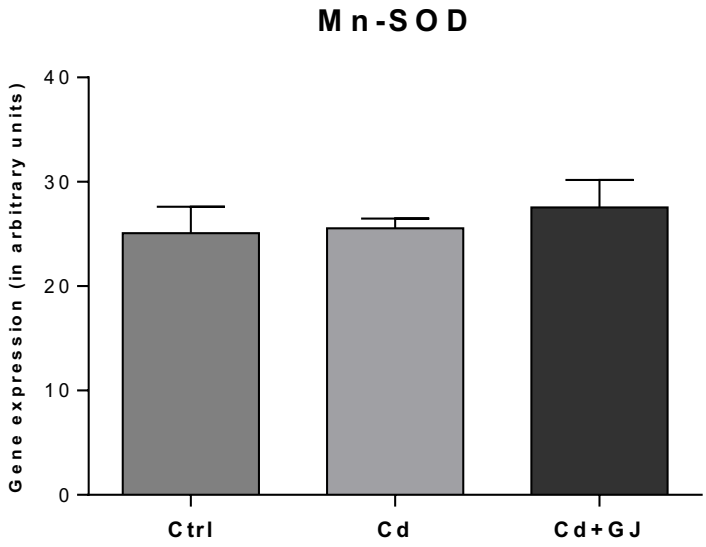

Fig. 7: Effects of grape juice concentrate G8000™ on the expression of Mn-SOD in cadmium liver of rats exposed to cadmium and treated with grape juice concentrate G8000™ using real-time PCR. ANOVA followed Tukey's multiple comparisons test. p>0.05. Cadmium group (Cd); CTRL control group, Cd+GJ cadmium and grape juice.

DISCUSSION

The aim of this study was to evaluate the antigenotoxic and antimutagenic potential of grape juice concentrate G8000™ in blood and liver of rats exposed to cadmium. To the best of our knowledge, the approach has not been addressed so far.

Micronucleus and comet assays are widely used tests for genotoxicity of potential substances as well as for detecting DNA damage as evidenced by strand breaks in various organisms and tissues (Çelik et al. 2009; The Comet Assay International Validation Management Team (2013)). DNA lesions induced by heavy metals consist of DNA single and/or double-strand breaks, DNA–DNA cross-links, DNA–protein cross-links, and base modifications (Yu et al. 2007; Anetor 2012). According to Hartwig (2010), the mechanism of genotoxicity of cadmium may be modulated by increased ROS generation; by interaction with the cellular DNA damage repair processes, or by epigenetic changes in DNA methylation.

The recognition of the antioxidant, antimutagenic, and anticarcinogenic polyphenols action has stimulated the research of these substances for treating, protecting, or even preventing several diseases (Ferguson 2001). Grapes contain many active phenolic compounds which can eliminate free radicals and prevent aggressive metabolites that act as mutagenic agents. Our results showed that grape juice concentrate G8000™ strongly modulated the genotoxic effects induced by single cadmiumexposure, preventing genetic damage induced by cadmium in blood and liver cells. Furthermore, the micronucleus test performed in bone marrow and liver showed that grape juice concentrate was clearly able to prevent chromosomal breakage caused by cadmium in blood and liver cells. According to Çelik et al. (2009), cadmium is a toxic substance in bone marrow at acute exposure and peripheral blood at chronic treatment. Their findings showed that acute and chronic cadmium treatment at 15 mg/kg dosage, for 24 h or 60 days, induced a raise on the number of micronucleated cells when compared with control group in bone marrow and peripheral blood cells. Palus et al. (2003) correlated cadmium concentration and genotoxicity in peripheral blood cells, showing that the incidence of micronucleus in these subjects was twice as high as the control group (not exposed to heavy metals). By comparison, Fahmi et al. (2013) investigated the antimutagenic activity in bone marrow mice cells of five different grape cultivar extracts and showed that all extracts had decreased

micronucleus incidence after endoxan treatment. Yalçin et al. (2010) treated mice with grape seed extract (at 50 and 150 mg/kg) in doxorubicin induced genotoxicity. Their results indicated a significant reduction in the frequency of micronucleated cells compared with the group treated with doxorubicin alone. This inhibition was dose dependent, being the total number of micronucleated cells lower in the group that received the highest dosage of grape seed extract.

Evidences suggest that either exogenous or endogenous agents can alter the cellular DNA as well as cellular components. Alkylating agents have been recognized as DNA damaging agents able to induce mutations in eukaryotic cells (Lundin et al. 2005). DNA damage caused by alkylating agents is predominantly repaired by the base excision repair pathway and DNA alkyl-transferases (Lindahl and Wood 1999). Some researchers support the idea that these compounds are the most potent and abundant chemical DNA damagers found in our environment (Freeman et al. 1994). Alkylation-induced genotoxicity assay is a suitable tool for evaluating DNA repair deficiency. Our results revealed that grape juice concentrate G8000™, at a dietary dose, was able to prevent alkylation-induced genotoxicity after cadmium exposure. These findings are new, and therefore difficult to discuss. Despite the biological mechanism involved in this phenomenon, we have demonstrated that grape juice concentrate G8000™was able to stimulate DNA repair systemin rats exposed to cadmium.

Hydrogen peroxide is a potent endogenous oxygen reactive species, and it is able to interact directly with DNA and through highly reactive oxygen and radical species to cause extensive oxidative DNA damage. Oxidative DNA damage has been recognized as a major cause of cell death and mutations in all aerobic organisms. In humans, oxidative DNA damage is also considered an important promoter of cancer. It is well known that micronucleus formation is linked to increased production of ROS, inhibition of DNA repair, as well as DNA breakage (Hengstler et al. 2003; Degrandi et al. 2010; Hartwig 2010; Bernhoft 2013). According to Aguiar et al. (2011), grape juice polyphenolic compounds were able to limit oxidative DNA damage promoted by free radicals. Our results also demonstrated that grape juice concentrate G8000™was able to prevent oxidative DNA damage in liver cells induced by hydrogen peroxide in vitro. Moreover, a decrease in immunoexpression of 8OHdG was detected in liver cells after treatment with grape juice concentrate. 8OHdG is an indicator of DNA damage, which

leads to adducts formation (Mikhailova et al. 1997; Filipič and Hei 2004; Rho and Kim 2006; Filipič 2012). Therefore, it seems that grape juice concentrate G8000™ acts as an antioxidant agent in liver cells.

It has been established that cadmium induces noxious biological effects such as a promoter of oxidative stress closely related to ROS elimination (Bertin and Averbeck 2006). Thus, it is fair to wonder about grape juice concentrate G8000™'s ability to modulate the antioxidant enzymes in this setting and to what extent. CuZn-SOD and Mn-SOD are powerful superoxide scavengers able to catalyze the conversion of O2*− radicals into O2 and H2O2 (Angermüller et al. 2009; Klaunig et al. 2010). Some published studies reveal different results regarding the activity of antioxidant enzymes in animals exposed to cadmium. Ognjanović et al. (2008) administered cadmium chloride (15 mg kg−1 day−1 for 4 weeks) to rats, and their results showed a decrease in liver SOD activity. Jihen et al. (2011) offered cadmium chloride in tap water (200 mg Cd/L) during 35 days to rats and observed an increase on CuZn-SOD activity, whereas Mn-SOD activity was not affected by cadmium.

Our data revealed that cadmiumwas not able to alter CuZn-SOD and Mn-SOD expressions. These results can be explained by Casalino et al.'s (2002), who demonstrated that CuZn-SOD expression became similar to the control group after 30 days of daily oral cadmium intake. Regarding the animals treated with grape juice concentrate, we observed an increase of CuZn-SOD levels. Conversely, the levels of Mn-SOD did not show remarkable differences in rats treated with grape juice concentrate G8000™. Such findings are fully in line with Jihen el et al. (2011), Thijssen et al. (2007), and Jurczuk et al. (2006).

In summary, our results demonstrated that grape juice was able to modulate the toxic effects caused by cadmium exposure as a result of its antigenotoxic and antimutagenic activities in blood and liver cells of rats.

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Conflict of interest: None declared.

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APPLE JUICE ATTENUATES GENOTOXICITY AND OXIDATIVE STRESS INDUCED BY CADMIUM EXPOSURE IN MULTIPLE ORGANS OF RATS

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ABSTRACT

The aim of this study was to evaluate the health benefits associated with apple consumption following cadmium exposure. A total of 15 Wistar rats were distributed into three groups (n=5), as follows: control group (non-treated group, CTRL); cadmium group (Cd) and apple juice group (Cd+AJ). The results showed a decrease in the frequency micronucleated cells in bone marrow and hepatocytes in the group exposed to cadmium and treated with apple juice. Apple juice was also able to reduce the 8OHdG levels and to decrease genetic damage in liver and peripheral blood cells. Catalase (CAT) was decreased following apple juice intake. Taken together, our results demonstrate that apple juice seems to be able to prevent genotoxicity and oxidative stress induced by cadmium exposure in multiple organs of Wistar rats.

INTRODUCTION

Over recent years, concern about environmental pollution as well as its impact on human health has been the subject of several studies so far [1–3]. It is well established that exposure to environmental pollutants is related with the etiology and pathogenesis of many chronic degenerative diseases [4].

Cadmium is one of the most toxic environmental and industrial pollutants, present in contaminated soil, water, air, foodstuffs and in the smoke released by cigarettes [5,6]. The metal is highly toxic, accumulating in various human tissues [7], and it induces oxidative stress, lipid peroxidation and DNA damage. Moreover, it is responsible for several pathologies such as renal dysfunction, skeletal disorders, cardiovascular diseases, lung, kidney and gastrointestinal damage, as well as liver and salivary glands malfunction and cancer [8–13].

In view of the human exposure to cadmium due to environmental, occupational, dietary and smoking contamination, the search for methods able to modulate the influence of this metal on the development of diseases is extremely important [4]. The current knowledge on the mechanisms of action of cadmium exposure in our body leads us to believe that the consumption of antioxidant compounds might be able to mitigate the toxic effects caused by the metal, either by scavenging of reactive oxygen species (ROS) or by the chelating action [14]. Therefore, it has been recommended the regular consumption of functional foods prevents some chronic degenerative diseases [15].

Apple and its derivatives are a great source of nutrients due to high levels of bioactive compounds such as polyphenols and phytochemicals [16]. Studies show that the consumption of apples is associated with decreased risk of developing chronic diseases [17]. According to Ribeiro et al. [18] and Soyalan et al. [19], apple and their compounds act as an antioxidant agent, improving the production of oxidative stress enzymes.

This work aimed to know the benefits of apple consumption following cadmium exposure as a result of its biological effects on genotoxicity and oxidative stress by gene expression of Copper-Zinc Superoxide Dismutase (CuZn-SOD), Manganese Superoxide Dismutase (Mn-SOD) and Catalase (CAT).

MATERIALS AND METHODS

Animals and experimental design

All experimental protocols involving animals are conformed to procedures described in the Principles for the Use of Laboratory Animals Guidelines. The study was approved by the Animal Ethics Committee of Federal University of Sao Paulo, UNIFESP, SP, Brazil (Protocol: CEUA number 484411).

A total of 15 Wistar rats weighing ~250 g, 8 weeks age were distributed into three groups (n = 5), as follows: Control group (nontreated group, CTRL); Cadmium group (Cd) and Apple Juice group (Cd + AJ). All animals were provided from Development Center ofExperimental Models for Medicine and Biology (CEDEME) of Federal University of São Paulo, SP, Brazil, and they were maintained under controlled conditions of temperature (23 ± 1 ^{o}C), light–dark periods of 12 h and free access to water and diet.

Animals from CTRL group received a single intraperitoneal (ip) water injection while those from the groups Cd and Cd + AJ received a single dose of 1.2 mg/kg body weight (BW) intraperitoneally (CdCl2, Nuclear ™), diluted in water according to the method described by Predes et al. [20]. After 15 days, Cd + AJ group received apple juice for 15 days, by gavage (1 mL, per day). CTRL and Cd groups were treated by gavage containing drinking water during the same experimental period. All animals were checked daily in order to evaluate individual behavior and general health conditions. The body mass was recorded weekly. No significant statistically differences ($p > 0.05$) in body weight were detected among groups after experimental design. Such data are demonstrated in Table1.

At the end of the experimental period, all animals were anesthetized with inhalational anesthetic halothane (Tanohalo™, Cristália™, SP, Brazil) and euthanatized for colleting tissues/organs to be used in this study. Previous studies have demonstrated that anesthesia does not interfere with redox status or cytokine profile levels [21,22].

The chemical characterization of apple juice (pulp wash juice) was performed in a previous study conducted by our research group [18]. The major components of

interest with m/z between 300 and 500 identified in it was caffeoylquinic acid, cyanidin-3-Oglucoside, 1-dodecanoyl-glycero-3-phospho-(10-sn-glycerol) and heptacosanoic acid. Several studies have calculated and estimated the daily amount of flavonoids that should be consumed by individuals. According Kahle et al. [23] and van der Sluis et al. [24], it is estimated that the daily amount of flavonoid intake should be between 0.15 and 1 g of total phenols. Under previous analysis, the apple juice used in this work has 4.3 mg/mL of total phenols. In this regard, it was administered 1 mL of apple juice daily.

Micronucleus test

After completing the experimental period, the micronucleus test was performed in bone marrow and liver tissue. The micronucleus test using bone marrow cells was performed according to Ribeiro et al. [25]. For this purpose, femoral bones were collected and stored in sodium chloride at 0.9%. The proximal epiphyses of bones were removed and 1 mL of fetal bovine serum (FBS; Cultilab™, Campinas, São Paulo, Brazil) was injected into the medullar canal. A smear on glass slide was performed with the suspension formed by the bone marrow and fetal bone serum. After drying the slides, they were stained with Giemsa (Merck™, Darmstadt, Germany). For liver, the micronucleus test was performed using paraffin sections (3 μm) stained by Feulgen and counterstained with Fast Green (Sigma Aldrich™, USA). A total of one thousand polychromatic erythrocytes or hepatocytes were analyzed per animal. Slides were scored blindly using a light microscope with a 100× immersion objective.

Single cell gel (Comet) assay

The protocol used for peripheral blood and liver cells followed the guidelines outlined by Tice et al. [26]. Peripheral blood was collected by cardiac puncture and liver cells were obtained by liver tissue maceration with PBS. Cells were transferred to individual plastic tubes, containing 1 mL of cold phosphate buffer solution (PBS, Ca^{+2}, Mg^{+2} free, pH 7.3), and centrifuged for 5 min, 1000 rpm, at room temperature. The supernatant was removed and the cell suspensions (~10 μL) were used for single cell gel (Comet) assay. A volume of 10 μL of cellular suspension was added to 120μL of

0.5% low-melting point agarose at 37 ºC, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and the slides immersed in lysis solution (2.5 mol L^{-1} NaCl; 100 mmol L^{-1} EDTAn – Merck™, Darmstadt, Germany; 10 mmol L^{-1}; Tris-HCl buffer, pH 10 – Sigma Aldrich™, St Louis, MO, EUA; 1% sodium sarcosinate – Sigma™, St Louis, MO, EUA; with 1% Triton X-100 – Sigma™, St Louis, MO, EUA; 10% dimethyl sulphoxide – Merck™, Darmstadt, Germany) for about 1 h. Afterwards, the slides were washed in ice-cold PBS (Ca^{+2}, Mg^{+2} free, pH 7.3) for 5 min, left in electrophoresis buffer (0.3 m mol L^{-1} NaOH and 1 m mol L^{-1}; EDTA - Merck™, Darmstadt, Germany, pH > 13) for DNA unwinding during 20 min, and electrophoresed in the same buffer for 20 min at 25 V (0.86 V/cm) and 300 mA. Following electrophoresis, slides were neutralized 0.4 mol L^{-1} Tris-HCl (pH 7.5, Sigma Aldrich™, St Louis, MO, EUA), fixed in absolute ethanol and stored at room temperature until analysis in a fluorescence microscope at 400× magnification. All steps were performed under reduced light.

A total of 50 randomly captured comets per animal (25 cells from each slide) were examined blindly by one expert observer at 400× magnification using a fluorescent microscope (Olympus, Orangeburg, NY, USA). The microscope was connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments™, Suffolk, Haverhill, UK) calibrated previously according to the manufacturer's instructions. To measure DNA damage, we used the tail moment defined as the product of the tail length and the fraction of DNA in the Comet tail [26].

Challenge assay

For this purpose, one aliquot (20 µL) from liver cells was treated with 4-nitroquinoline 1-oxide (4NQO, 0.05 m mol L^{-1}) for 15 min or H_2O_2 (0.6 m mol L^{-1}) for 5 min. Then, agarose was added and smears were made and left to lysis solution (2.5 mol L NaCl, 100 m mol L^{-1} EDTA, 10 mmol L^{-1} Tris–HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100, and 10% DMSO) for about 1 h. All the slides prepared were immersed into alkaline buffer for 20 min and electrophoresis was conducted for another 20 min, at 300 mA and 25 V. After that, the slides were

neutralized using Tris solution (Sigma Aldrich™, EUA) and fixed at ethanol and stored at room temperature until analysis in a fluorescence microscope at 400× magnification. The analysis was the same described previously.

8-OHdG immunohistochemistry

Liver serial sections of 4 µm were desparafinized in xylene and rehydrated in graded ethanol (99.5%), then pretreated in a microwave with 10 m mol L^{-1} citric acid buffer (pH 6, 0.1 mol L^{-1} citric acid - Synth™, SãoPaulo, Brazil; 0.1 mol L^{-1} sodium citrate - Synth™, São Paulo, Brazil) for 3 cycles of 5 min each for antigen retrieval. They were pre-incubated with 0.3% hydrogen peroxide for inactivation of endogenous peroxidase and then blocked with 5% normal goat serum for 30 min. The specimens were then incubated with anti-8-hydroxy-20-deoxyguanosine (8OHdG, Santa Cruz Biotechnologies Inc™, MO, USA) at 1:100 dilution, overnight, at 4 °C.This was followed by two washes in PBS and further incubation with a biotinylated secondary antibody, diluted 1:100 in PBS for 1 h. The sections were washed twice with PBS followed by the application of preformed avidin biotin complex (Vector Technologies™, USA) for 45 min. The bound complexes were visualized by the application of a 0.05% solution of 3,3-diaminobenzidine (Sigma™, St Louis, MO, EUA) and counterstained with hematoxylin (Sigma™, St Louis, MO, EUA). Sections stained using immunohistochemistry were analyzed for the percentages of immunopositive cells in liver. A total of 1000 hepatocytes were evaluated in 3-5 fields at 400× magnification. These values were used as labeling indices.

Real time PCR

Liver tissue at -86 °C was homogenized and total RNA was isolated using cold Trizol Reagent (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was determined using a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies™, Wilmington, DE). RNA samples were treated with DNAse (DNase Amplification Grade™, Applied Biosystems™, Foster City, CA, USA) to avoid contamination with genomic DNA.

cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed in the 7500 Fast Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA) using the Power SYBR Green Kit (PCR Master Mix 2x, Applied Biosystems™, Foster City, CA, USA). Primers for the specific amplification of each cDNA were designed using the Primer Express software (Applied Biosystems™, Foster City, CA, USA), considering established criteria, such as product length, optimal PCR annealing temperature and the likelihood of primer self-annealing. The primers sequence are: GAPDH: sense 5' - CAA CTC CCT CAA GAT TGT CAG CAA - 3' and anti-sense 5' – GGC ATG GAC TGT GGT CAT GA – 3'; CuZn-Superoxide Dismutase: sense 5' – CCAGTGCAGGACCTCATTTT – 3' and anti-sense 5' – CCT TTC CAG CAG TCA CAT TG – 3'; Manganese-SOD: sense 5' - AAC ATT AAC GCG CAG ATC A – 3' and anti-sense 5' - AAT ATG TCC CCC ACC ATT GA-3';Catalase: sense 5' – AGC GGA TTC CTG AGA GAG TG – 3' and anti-sense 5' – GAG AAT CGA ACG GCA ATA GG – 3'.

PCR reactions were performed in duplicate containing 20 µL final volume using 2.0 µL of a 1:5 (v/v) dilution of cDNA, 2.0 µL primer mix (forward and reverse), 10.0 µL of Power SYBR Green™ (PCR Master Mix 2x™, Applied Biosystems™, Foster City, CA, USA) and DPEC water™ (Ultrapure DEPC Treated Water™, Invitrogen™, Carlsbad, CA, USA). The reactions were performed in Micro Amp 96-well plates™ (Applied Biosystems™, Foster City, CA, USA) covered with optical adhesive (Applied Biosystems™, Foster City, CA, USA). Samples were submitted to forty cycles of 95 ^{o}C for 10 min, 95 ^{o}C for 15 s and 60 ^{o}C for 1 min. An amplification efficiency curve using different cDNA dilutions was also performed for each gene tested.

To normalize the data for the control and experimental groups, arbitrary units were calculated as: arbitrary unit = 2 -ΔΔCT, and ΔΔCT = sample ΔCT – control ΔCT, where CT is the threshold cycle.

Statistical analysis

All the data are expressed as mean ± standard deviation (SD). For Micronucleus Test, immunohistochemistry and Real Time PCR data, it was used one-way analysis

of variance (one way-ANOVA) followed Tukey's multiple comparisons test. For Comet Assay, two-way analysis of variance (two way-ANOVA) was performed followed by Tukey's multiple comparisons test. Statistical analysis was performed using Graph Pad Prism™ 6.0 program. $p < 0.05$ was considered to be significant.

RESULTS

Micronucleus test

The micronucleus test data in bone marrow and liver showed that cadmium was able to induce the formation of micronuclei in both tissues. A significant decrease in the number of micronucleated erythrocytes and hepatocytes was detected in animals exposed to cadmium and treated with apple juice (Cd+AJ) when compared to animals exposed to cadmium (Cd) only (Figs. 1 and 2).

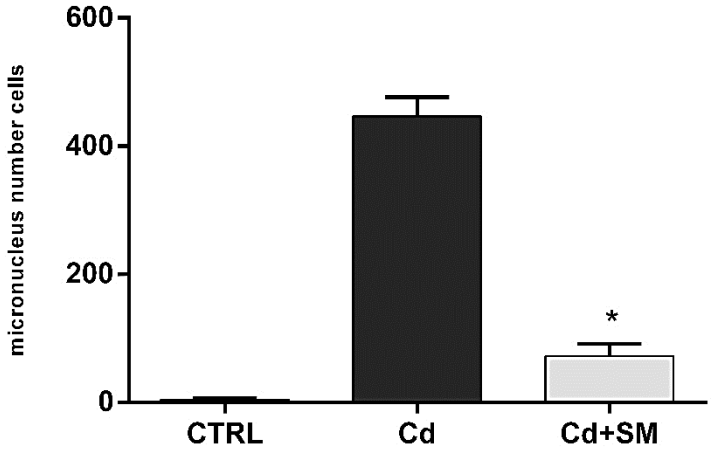

Fig. 1. Micronucleus number cells in bone marrow of animals cadmium-exposed and treated with apple juice. One-way ANOVA followed Tukey's multiple comparisons post hoc. *Compared to cadmium group (Cd). $p<0.0001$; Cd+AJ, cadmium plus apple juice group.

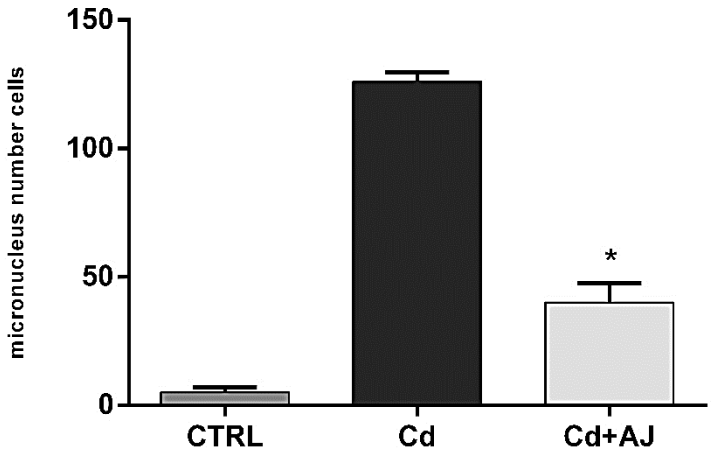

Fig. 2. Micronucleus number cells in hepatocytes of animals cadmium-exposed and treated with apple juice. One-way ANOVA followed Tukey's multiple comparisons post hoc. *Compared to control group (CTRL); **compared to cadmium group (Cd). $p<0.0001$; Cd+AJ, cadmium plus apple juice group.

Comet assay

Regarding genotoxicity, liver showed an increase in the tailmoment values for cadmium group (Cd) when compared to control group (CTRL), whereas the group treated with apple juice (Cd+AJ) showed a significant reduction for DNA damaging. Peripheral blood showed a significant increase for genetic damage in the Cd group when compared to the CTRL group. A significant reduction for DNA damage in Cd+AJ was noticed. The results from Single Cell Gel (Comet) Assay are shown in Fig. 3a. In this study, we also evaluated the mutagen sensitivity induced by hydrogen peroxide (H_2O_2) or 4-nitroquinoline 1-oxide (4-NqO), well-known mutagenic agents, in rats exposed to cadmium andapple juice. Our results demonstrated that apple juice was able to protect the liver against the oxidative stress induced by H_2O_2 as well as the alkylation damage induced by 4NQO. Such findings are demonstrated in Fig. 3b.

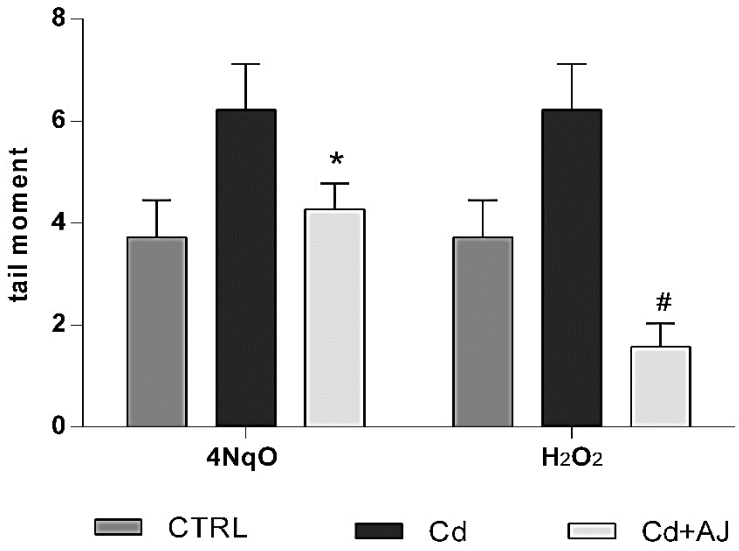

Fig. 3. (A) Comet assay: DNA damage in multiple in peripheral blood and liver of rats exposed to cadmium and treated with apple juice. **(B)** Challenge assay: DNA damage in hepatocytes of rats exposed to cadmium and treated with apple juice and exposed to 4NqO and H_2O_2. Two-way ANOVA followed Tukey's multiple comparisons post hoc.*Compared to respective cadmium group (Cd); #compared to respective cadmium group (Cd). $p<0.0001$; CTRL, control group; Cd+AJ, apple juice plus cadmium.

Immunohistochemistry

Immunohistochemical data revealed a decrease to 8OHdG immunoexpression in rat hepatocytes exposed to cadmium and treated with apple juice (Cd+AJ). Apple juice was able to reducethe 8OHdG levels when compared to cadmium group (Cd), being significant statistically differences ($p<0.05$) between groups. These results are summarized in Figs. 4 and 5.

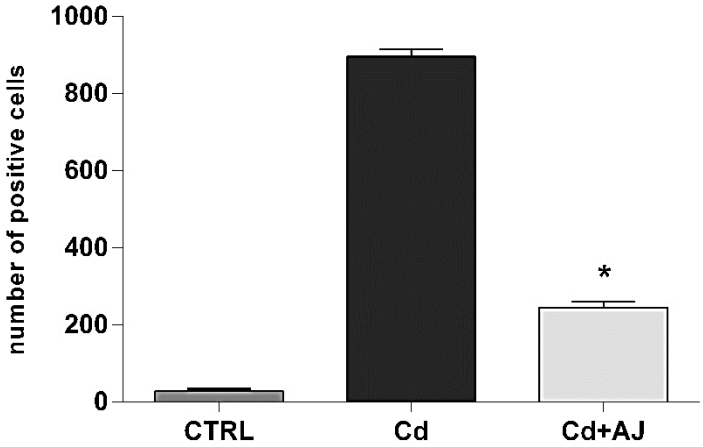

Fig. 4. Total number of immunopositive cells for 8-OHdG. One-way ANOVA followed Tukey's multiple comparisons post hoc. *p<0.0001 when compared with cadmium group (Cd). Cd+AJ, apple juice plus cadmium group.

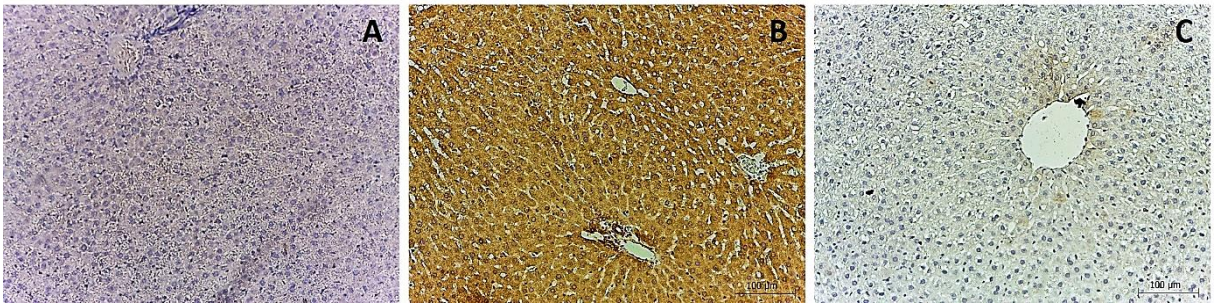

Fig. 5. Immunohistochemistry for 8OHdG: **(A)** control group (CTRL); **(B)** cadmium group (Cd); **(C)** apple juice plus cadmium group.

Real time PCR

Antioxidant gene expression of Copper-Zinc Superoxide Dismu-tase (CuZn-SOD), Manganese Superoxide Dismutase (Mn-SOD) and Catalase (CAT) were analyzed by Real Time Polymerase Chain Reaction (qPCR). CuZn-SOD and Mn-SOD gene expression did not show significant statistically differences (p>0.05) between groups (Fig. 6a and b, respectively). Nevertheless, catalase expression decreased in Cd+AJ group when compared to CTRL and Cd groups (p<0.05; Fig. 6c).

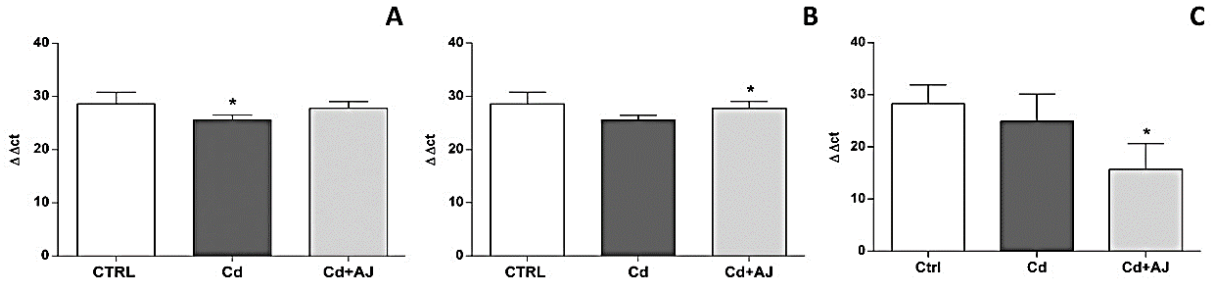

Fig. 6. Effects of apple juice on the gene expression of **(a)** CuZn-SOD; **(b)** Mn-SOD and **(c)** catalase in liver of rats exposed to cadmium and treated with apple juice, using real-time PCR. One-way ANOVA followed Tukey's multiple comparisons test. *p<0.05 when compared to CTRL. **p<0.05 when compared to cadmium group (Cd) or CTRL groups.

Discussion

The concern about the possible development of diseases from pollutants is increasing day by day. Among the innumerable toxic substances to which we are exposed daily is cadmium, a heavy metal whose half-life ranging from 10 to 30 years. It promotes extensive damage to several tissues such as liver, kidney, lungs and blood [27, 28]. Furthermore, cadmium is able to induce DNA damage by means of interactions with the cellular DNA damage response system and by the production of ROS which promotes lipid peroxidation as far as DNA breakage [29, 30].

Many food compounds may exert antioxidant action by scavenging free radicals and metal chelating [31, 32]. Apple and their derivatives have shown important antioxidant activity [33, 34] due to the presence of flavonoids (catechins, flavonols, quercetin), phenolic acids (quercetin glycosides, catechin, epicatechin, pro-cyanidins), vitamins and fibers [15], which have been described as contributors to human health against cardiovascular diseases, asthma and pulmonary dysfunction, diabetes, obesity and cancer [35].

The micronucleus test and Comet assay showed that cadmium was able to induce the formation of micronucleus and DNA damage, respectively. These assays are widely employed to detect the genotoxicity of xenobiotics as a result of DNA strand breaks that could be induced by oxidative stress [36, 37]. According to Tapisso et al.

[38], the presence of cadmium is related with increased frequencies of micronucleus and, even at low concentrations, is capable to induce DNA damage.

Protection of DNA damage as well as modulation of DNA repair system play important role on mutagenesis [39]. Several fruits and vegetables, as well as its compounds, have been investigated fortheir antioxidant effects [40]. Apple compounds possess a wide range of biological activities which may contribute to human health due to antimutagenic and antioxidant activities, anti-inflammatory action, modulation of signal transduction pathways and carcinogen metabolism, antiproliferative and apoptosis-inducing activity [16]. In this study, it was observed that apple juice was able to reduce the number of micronucleated cells induced by cadmium exposure in erythrocytes and hepatocytes. Probably, such effect was caused by antioxidant activity of apple juice. When hepatocytes were exposed to oxidative and alkylating agents, apple juice was able to inhibit DNA damage induced by previous exposure to cadmium in vivo. In a study conduct by Wilms and colleagues [41], quercetin, which is one of the major compounds founded in apples, it is very effective for preventing induced oxidative DNA damage. Moreover, it can reduce genetic damage induced by mutagenic agents in a dose-dependent manner in vitro.

The interaction of reactive oxygen species with DNA molecule leads to the formation of 8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2'-deoxyguanosine), leading to the generation of radical adducts and synthesis of 8-hydroxy-2'-deoxyguanosine (8-OHdG), one of the most important marker for measuring endogenous oxidative damage to DNA [42]. Our results demonstrated that cadmium exposed group displayed increased 8-OHdG immunoexpression in liver cells.

The literature describes that cadmium induces oxidative damage by disturbing the antioxidant defense systems, and the enhancement of ROS production is responsible to suppress free-radical scavengers enzymes, such as SOD and CAT [43-46]. In a study performed in Turkey showed that apple attenuates oxidationimproving the concentration of antioxidant enzymes such as SOD inerythrocytes and plasma of humans who consumed fresh apples fora month. Such data suggest that apple intake increases antioxidant enzymes levels by diminishing oxidative stress [47]. Our results revealed that apple juice did not alter CuZn-SOD and Mn-SOD levels to the animals

exposed to cadmium. Nevertheless, apple juice group decreased catalase expression in cadmium intoxicated rats. This result could be explained by the fact that the mechanism of action of polyphenols is related to metals removal as well as scavenging free radicals [31]. The inactivation of these elements occurs when an antioxidant interacts with the free radical even when there action leads to formation of a less reactive metal [48]. So, low levels of the enzyme catalase activity in this study may be explained by the action of polyphenols present in apple without the need to activate some antioxidant enzymes. In summary, our results demonstrate that apple juice seems to be able to prevent genotoxicity and oxidative stress induced by cadmium exposure in multiple organs of rats.

Conflict of interest

None declared.

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6 DISCUSSÃO

A preocupação com uma possível relação entre o mecanismo de desenvolvimento de doenças e poluentes ambientais cresce a cada dia. Dentre as inúmeras substâncias tóxicas às quais estamos expostos diariamente está o cádmio, um metal pesado cuja meia-vida encontra-se em uma faixa entre 10 e 30 anos, acumula-se no organismo e promove extensos danos a vários tecidos como fígado, rins, pulmões e sangue (Nair et al. 2013; Wang et al. 2013; Anetor 2012).

Diversos estudos relataram que a principal fonte de contaminação por cádmio é pela fumaça de cigarro e alimentos contaminados, visto que a contaminação de solo por metais pesados tem aumentado continuamente no último século (Ellis et al. 2012; Satarug et al. 2011; Järup e Åkesson, 2009). A Organização de Alimentos e Agricultura da OMS determina que a ingestão semanal tolerável de cádmio seja de 7 µg/kg de peso corporal. Segundo relatório da OMS, a média de ingestão de cádmio varia consideravelmente de 10 a 40 µg em áreas não-poluídas e de algumas centenas de microgramas em áreas contaminadas (Järup et al. 1998).

A absorção do cádmio é dependente da forma de exposição ao metal. Dados apontam que a via respiratória apresenta alta absorção de cádmio, com taxas de até 40%; enquanto que a absorção via TGI é de apenas 2% (Świergosz-Kowalewska 2001). Em trabalho de revisão da literatura, Adikwu et al. (2013) afirmaram que, independente da via de contato com o cádmio, seja oral, intraperitoneal, subcutânea ou intravenosa, todas são indutoras de danos hepatotóxicos. Świergosz-Kowalewska (2001) assumiram que a concentração de cádmio crítica ao fígado e rins varia entre 20-30 µg/g e 100-200 µg/g, respectivamente. Em humanos, estudos realizados por Satarug e colaboradores (2003 e 2000) revelaram que o consumo diário de 25-30 µg é deletério para os rins.

A genotoxicidade do cádmio ocorre, principalmente, pelo aumento de EROs gerado pelo desequilíbrio das enzimas antioxidantes, promovendo oxidação do DNA, lipídios e proteínas (Matović et al. 2011; Nemmiche et al. 2011). Consequentemente, danos ao material genético podem levar à parada do ciclo celular e instabilidade genômica, além de interferir no sistema de reparo de DNA (Hartwig 2010; Bertin e Averbeck, 2006).

Até o momento, não exite um consenso na literatura com relação a possíveis tratamentos para intoxicação por cádmio, especialmente em populações que vivem em areas contaminadas (Bernhoft 2013; Matović et al. 2011). De acordo com Flora e Pachauri (2010), a maioria dos quelantes utilizados para a remoção de metais, a citar EDTA e seus derivados, BAL (dimercaprol), DMSA (ácido meso 2,3-dimercaptosucinico), DFOA (desferrioxamina), dentre outros, possuem inúmeros efeitos adversos. Particularmente ao cádmio, estudos revelaram que o uso de algumas substâncias quelantes poderiam aumentar a reabsorção do metal pelos rins aumentando os danos celulares (Prozialeck e Edwards 2012). Assim, a busca por novas estratégias para o tratamento de intoxicação por metais têm sido alvo de diversos grupos de pesquisa no país e no mundo.

O uso de substâncias antioxidantes oriundas da dieta pode ser uma alternativa promissora para a prevenção ou tratamento dos danos causados pelo cádmio. Produtos naturais provenientes da dieta proporcionam um grande número de substâncias antioxidantes (Sen et al. 2010). O reconhecimento da ação antioxidante, antimutagênica e anticarcinogênica dos polifenóis tem estimulado a pesquisa dessas substâncias para o tratamento, proteção e prevenção de diversas doenças (Fegurson 2001). Estes compostos, presentes em muitos alimentos, exercem atividade antioxidante por meio do sequestro de radicais livres e ação quelante (Flora et al. 2013, Fraga et al. 2010).

Uva e maçã contêm muitos compostos fenólicos ativos cuja ação é neutralizar os radicais livres, previnindo a mutagenicidade e contribuindo para o surgimento de doenças cardiovasculares e respiratórias, diabetes, obesidade e câncer (Kujawska et al. 2011). O consumo de suco de uva ou de maçã por duas semanas elevam a capacidade antioxidante total plasmática e diminuem a concentração de malondialdeído, um produto da peroxidação lipídica, além de elevar os níveis das enzimas antioxidantes (Yuan et al. 2011).

Nosso grupo de pesquisa gerou resultados positivos no estudo do efeito nutracêutico dos sucos de uva e maçã em diversos modelos experimentais, comprovando a sua eficácia contra os danos inflamatórios, oxidativos e mutagênicos observados na colite experimental (Marchi et al. 2014; Paiotti et al. 2013), esteatose hepática não alcoólica (Aguiar Jr. 2011), câncer (de Jesus et al. 2014; Ribeiro et al. 2014; Ribeiro et al. 2015), nos efeitos tóxicos gerados pelo cádmio no aparelho

reprodutor (Pires et al. 2013) e, recentemente, sobre o perfil lipídico em seres humanos dislipidemicos (Peres et al. 2015). Assim, mantendo esta linha de pesquisa, este trabalho hipotetizou se o concentrado do suco de uva G8000® e o suco de maçã seriam capazes de promover efeitos benéficos contra a citotoxicidade, mutagenecidade e estresse oxidativo promovidos pela exposição ao cádmio em sangue e fígado.

O teste do Micronúcleo e o Ensaio do Cometa são técnicas de análise amplamente utilizadas para investigar o potencial genotóxico das mais diversas substâncias e produtos, além de detectar evidências de danos ao DNA em vários órgãos e tecidos (Çelik et al. 2009; The Comet Assay International Validation Management Team, 2013). De acordo com Tapisso e colaboradores (2009), a exposição ao cádmio gera um aumento na frequência de micronúcleos e, mesmo em baixas concentrações, é capaz de induzir danos genéticos. Os resultados aqui apresentados mostraram que tanto o concentrado de suco de uva G8000® como o suco de maçã foram capazes de modular esta resposta, promovendo uma ação anti-genotóxica, a partir da diminuição de quebras de fitas simples e duplas no DNA e quebras cromossômicas em células sanguíneas e hepatócitos.

De acordo com Çelik et al. (2009), a exposição aguda (24 horas) ou crônica (60 dias) de 15 mg/kg de cádmio induz um aumento no número de células micronucleadas em sangue periférico e medula ossea. Pallus et al. (2003) correlacionaram a concentração de cádmio e a genotoxicidade em células de sangue periférico, mostrando que a incidência de micronúcleo é duas vezes maior que em células não expostas ao metal.

A partir de avaliação da atividade antimutagênica de cinco espécies de uvas em células de medula óssea de camundongos, os resultados apontaram que todos os extratos utilizados diminuíram a incidência de micronúcleos, após tratamento prévio com endoxan (ciclofosfamida) (Fahmi et al. 2013). Yalçin e colaboradores (2010) avaliaram os efeitos de extrato de semente de uvas (a 50 e 150 mg/kg) em camundongos expostos ao doxorubicina e os resultados indicaram uma significativa redução na frequência de células micronucleadas no grupo exposto à droga e tratados com um dos extratos que, segundo os autores, apresentaram uma resposta de inibição tipo dose-dependente.

Compostos derivados de maçã possuem uma grande variedade de biocompostos que contribuem com à saúde graças aos seus efeitos antioxidante e antimutagênico, bem como atividade anti-inflamatória, antiproliferativa, apoptótica, além de modular vias de transdução de sinais, eventos epigenéticos e a imunidade inata (Gerhauser 2008, Ribeiro et al. 2014). Asita e Molise (2011) mostraram que camundongos tratados previamente com suco de maçã e expostos à ciclofosfamida apresentaram diminuição no número de micronúcleos em eritrócitos policromáticos de medula óssea. Em estudo conduzido por Wilms e colaboradores (2005), a quercetina, que reconhecidamente é o principal composto bioativo presente na maçã, mostrou-se bastante eficaz na prevenção de danos oxidativos ao DNA.

A proteção ao dano genético e a modulação do reparo do DNA possuem um papel importante na prevenção de eventos mutagênicos (Ramos et al. 2011). Diversas frutas e vegetais, assim como seus biocompostos, têm sido investigados por suas ações antioxidantes (Hamid et al. 2010). Evidências sugerem que agentes exógenos ou endógenos podem alterar a composição celular. Agentes alquilantes são reconhecidos como substâncias capazes de induzir mutações em células eucarióticas (Lundin et al. 2005). Alguns pesquisadores afirmam que esses compostos estão entre as mais potentes e abundantes substâncias encontradas no ambiente capazes de promover dano cromossômico (Freeman et al. 1994).

A análise de genotoxicidade induzida por agentes alquilantes é uma ferramenta utilizada para avaliar a deficiência de reparo do DNA. Os dados resultantes dos Testes do Cometa e do Desafio revelaram que o concentrado de suco de uva G8000® e o suco de maçã são capazes de prevenir possíveis danos genotóxicos induzidos em ratos expostos ao cádmio, prevenindo danos oxidativos em células hepáticas. Portanto, nossos resultados demonstram que ambos os sucos são capazes de estimular o sistema de reparo de DNA, após a exposição ao cádmio.

O peróxido de hidrogênio é uma potente ERO endógeno que interage diretamente com o DNA e promove extenso dano oxidativo ao material genético. A oxidação do DNA é uma das principais causas de morte celular e mutações em organismos aeróbicos. Em humanos, a lesão oxidativa é considerada um importante promotor do câncer. É sabido que a formação de micronúcleos está associada ao aumento da produção de EROs, inibição do sistema de reparo de DNA e quebra

cromossômica (Hengstler et al. 2003; Degrandi et al. 2010; Hartwig 2010; Bernhoft 2013).

A interação de EROs com os pares de bases do DNA, como a guanina, é responsável pela formação da 8-hidroxiguanina (8-OHGua), levando à formação de adutos e, por conseguinte, à síntese de 8-hidroxi-2'-deoxiguanosina (8-OHdG), um dos mais importantes marcadores de estresse oxidativo endógeno (Filipič 2012; Valavanidis et al. 2009; Rho e Kim 2006; Filipič e Hei 2004; Mikhailova et al. 1997). Esta ação foi observada nos resultados obtidos nesse estudo na análise imunoistoquímica de 8-OHdG, visto que os animais do grupo exposto somente ao cádmio apresentaram uma forte imunoexpressão para este marcador e, quando expostos ao metal e tratados com concentrado de suco de uva G8000® ou suco de maçã reduziram expressivamente a imunomarcação.

Adicionalmente, o presente estudo observou que o concentrado de suco de uva G8000® e o suco de maçã possuem ação sobre as enzimas antioxidantes. O cádmio induz efeitos biológicos nocivos resultantes do estresse oxidativo gerado pela presença de EROs (Bertin e Averbeck 2006) promovendo, assim, um desequilíbrio do sistema de defesa antioxidante, suprimindo a ação de enzimas antioxidantes SOD e CAT (Cichoz-Lach e Michalak 2014; Nair et al. 2013; Filipič 2012, Matović et al. 2011; Nemmiche et al. 2011). As enzimas SOD-CuZn e SOD-Mn são potentes sequestradores de EROs que catalisam a conversão do ânion superóxido O_2^- em O_2 e H_2O_2 que, por sua vez, pode ser convertido no radical livre hidroxila, interagindo com outras moléculas biológicas e consequente síntese de outras EROs (Klaunig et al. 2010; Angermüller et al. 2009).

Alguns estudos revelaram resultados dissonantes quanto a atividade das enzimas antioxidantes em animais expostos ao cádmio. Ognjanović et al. (2008) administraram cloreto de cádmio (15 mg/dia/4 semanas) em ratos e mostraram um aumento na atividade da SOD. Jihen et al. (2011) ofertaram cloreto de cádmio (200 mg/L) na água de beber durante 35 dias em ratos e observaram um aumento na atividade de SOD-CuZn, entretanto, os níveis de SOD-Mn mostraram-se inalterados. Casalino et al. (2002), demonstraram que os níveis de SOD-CuZn em animais expostos ao cádmio, após 30 dias de exposição se tornam similares ao do grupo controle.

Com relação aos nossos resultados, o concentrado de suco de uva G8000® aumentou a expressão da enzima SOD-CuZn, mas os níveis de SOD-Mn permaneceram inalterados, corroborando com os resultados de Jihen et al (2011), Thijssen et al. (2007) e Jurczuk et al. (2006). Já o suco de maçã não apresentou quaisquer alterações na atividade dessas enzimas. Este resultado pode ser explicado pelo fato de que o mecanismo de ação dos polifenóis estar relacionado não só com o sequestro de radicais livres, mas também com a remoção de metais e a inativação destes elementos por meio da formação de complexos inertes não interfere na atividade das enzimas antioxidantes (Flora et al. 2013; Leopoldini et al. 2011; Fraga et al. 2010). Assim, o baixo nível de expressão da enzima CAT avaliada nos animais tratados com suco de maçã também pode ser explicado por este princípio, já que os polifenóis presentes no fruto poderiam ter agido diretamente no sequestro dos radicais livres sem a necessidade de ativação das mesmas.

Em suma, podemos afirmar que tanto o concentrado de suco de uva G8000® como o suco de maçã aqui utilizados apresentaram efeitos benéficos ao organismo murino quando exposto ao cádmio. Isso está respaldado em estudos prévios que mostram os benefícios das substâncias antioxidantes presentes em frutos e seus derivados, especialmente em uvas e maçãs (de Moura et al. 2013; Del Rio et al. 2013; Nile et al. 2013; Rho e Kim 2006; Boyer e Liu 2004). Mesmo ambos os sucos sendo ricos em polifenóis, cada qual apresenta tipos diferentes de biocompostos na sua composição. Estudos mais aprofundados deverão ser realizados a fim de compreender qual ou quais compostos poderiam ser responsáveis por tais ações ou verificar se o efeito antioxidante é proveniente do efeito sinérgico dos vários tipos de fenóis. Compreender o motivo pelo qual a oferta de uma menor concentração de polifenóis presentes na dose administrada no suco de maçã foi capaz de apresentar resultados semelhantes aos obtidos com o concentrado de suco de uva G8000® são de grande valia para se mensurar qual a quantidade exata de polifenóis necessária para obter um efeito benéfico a saúde humana.

7. CONCLUSÃO

Nossos resultados demonstram que o concentrado de suco de uva G8000® e o suco de maçã são capazes de modular a mutagenicidade e o estresse oxidativo induzidos pela exposição ao cádmio graças à presença de polifenóis e suas ações antioxidantes.

8 Anexo – Carta de aprovação do projeto de pesquisa pela Comissão de Ética no Uso Animal (CEUA)

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1933

COMITÊ DE ÉTICA EM PESQUISA

SP

São Paulo, 22 de janeiro de 2014
CEUA N 484411

Ilmo(a). Sr(a).
Pesquisador(a): Carolina Foot Gomes De Moura
Depto/Disc: Patologia
Daniel Araki Ribeiro (orientador)

Título do projeto: "Avaliação da ação dos concentrados de suco de uva e de suco de maçã em múltiplos órgaos de ratos expostos ao cloreto de cádmio".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais UNIFESP/HSP

O cádmio é um importante poluente industrial e ambiental (Edwards et al., 2012; Prozialeck et al., 2009). Estudos revelam que o cádmio, é absorvido pela via pulmonar ou pelo trato gastrointestinal (Nordberg, 2009; Sánchez-González et al., 2006). Quando absorvido pelo fígado, inibe enzimas hepáticas promovendo aumento da peroxidação lipídica, congestão, isquemia e hipóxia do órgão (Ramesh e Satakipan, 2010). Nos pulmões, a absorção e o acúmulo de cádmio são responsáveis pelo desenvolvimento de doenças como enfisema, bronquite e câncer (Ezzat et al., 2009; Panjehpour e Bayesteh, 2008). Pesquisas demonstram que o cádmio também possui efeito osteotóxico (Engström et al., 2011; Nawrot et al., 2010; Schutte et al., 2008). Independente do tecido afetado, a toxicidade desencadeada pelo cádmio é inicialmente caracterizada por danos oxidativos (Eybl et al, 2006). Grandes esforços têm sido feitos na tentativa de encontrar seguros e potentes antioxidantes de origem vegetal (Ramesh e Satakipan, 2010). Considerando os benefícios de substâncias antioxidantes e sua eficácia em reduzir os danos causados por metais pesados já descritos na literatura, propõe-se avaliar os efeitos do concentrado de suco de uva e do suco de maçã, ricos polifenóis em múltiplos órgãos de roedores submetidos à intoxicação aguda com cloreto de cádmio.

PARECER ANTERIOR: Em parecer anterior foi solicitada a adequação da dose do anestésico para eutanásia. Em resposta, o pesquisador alterou o tipo de anestésico a ser utilizado.

ANIMAIS: Serão utilizados:
30 Ratos heterogênico Wistar, Machos, com idade de 90 dias
Procedência: Biotério/Cedeme
Manutenção: Guarda de animais/Campus Baixada Santista

VIGÊNCIA DO ESTUDO: início previsto para: 12/2013 com término previsto para: 30/08/2015

A Comissão de Ética no Uso de Animais da Universidade Federal de São Paulo/Hospital São Paulo, na reunião de 09/01/2014, **ANALISOU** e **APROVOU** todos os procedimentos apresentados neste protocolo.
1. Comunicar toda e qualquer alteração do protocolo.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do protocolo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.
4. **Relatórios parciais** de andamento deverão ser enviados **anualmente** à CEUA até a conclusão do protocolo.

Rua Botucatu, 572, 1º andar conjunto 14 CEP 04023-061 São Paulo - tel: 55 (11) 5571-1062 / fax: 55 (11) 5539-7162
Horário de atendimento: das 9:00 as 13:00hs : e-mail:cepunifesp@unifesp.br

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COMITÊ DE ÉTICA EM PESQUISA

Atenciosamente,

Prof. Dr. José Osmar Medina Pestana
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de São Paulo/Hospital São Paulo

Rua Botucatu, 572, 1º andar conjunto 14 CEP 04023-061 São Paulo - tel: 55 (11) 5571-1062 / fax: 55 (11) 5539-7162
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