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Bruna da Cruz Weber Fulco

CISPLATINA INDUZ UM PADRÃO DE TOXICIDADE HEPATORRENAL DIFERENTE ENTRE RATOS NEONATOS E ADULTOS

Santa Maria, RS

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Dissertação apresentada ao curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Mestre em Bioquímica Toxicológica**

Orientador: Prof. Dr. Gilson Rogério Zeni

Coorientadora: Prof.ª. Dr.ª Cristina Wayne Nogueira

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2018

Dedico esta dissertação a minha família, principalmente ao meu irmão Gustavo que me acompanhou em cada etapa desta trajetória e compartilhou de cada momento de angústia, ansiedade e felicidade.

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RESUMO

CISPLATINA INDUZ UM PADRÃO DE TOXICIDADE HEPATORRENAL DIFERENTE EM RATOS NEONATOS E ADULTOS

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A cisplatina é um quimioterápico amplamente utilizado para tratar diversos tipos de tumores, tanto em adultos quanto em crianças. Apesar de bastante difundido, o uso da cisplatina está relacionado a efeitos tóxicos graves, dentre eles a nefrotoxicidade e a hepatotoxicidade. Desta forma, a presente dissertação investigou os efeitos causados pela administração aguda de cisplatina em ratos com 10 dias e as diferenças na resposta ao fármaco, no que se refere à toxicidade, quando comparada a ratos de 60 dias. Para a realização deste modelo experimental foram utilizados ratos Wistar machos e fêmeas, com 10 e 60 dias (CEUA nº 2699300315). Os animais receberam a cisplatina nas doses de 5 e 10 mg/kg pela via intraperitoneal (i.p.), enquanto o grupo controle recebeu a solução salina (0,9%; i.p.). Após 24 h da administração de cisplatina/salina, os animais foram mortos e o sangue, o fígado e os rins foram coletados para as análises *ex vivo*. Nas amostras de soro foram avaliados os parâmetros de dano renal (ureia) e hepático (atividade das enzimas alanina aminotransferase, aspartato aminotransferase). Enquanto que nas amostras de fígado e rim foram determinados: os marcadores de estresse oxidativo (níveis de espécies reativas de oxigênio, peroxidação lipídica, carbonilação de proteínas e tióis não-proteicos (NPSH)); e as atividades das enzimas antioxidantes (superóxido dismutase, catalase (CAT), glutationa peroxidase, glutationa-S-transferase e glutationa redutase); assim como as atividades das enzimas sulfidrílicas δ-aminolevulinato desidratase (δ-ALA-D) e Na^+, K^+- ATPase. Os níveis renais e hepáticos das proteínas relacionadas com o estresse oxidativo e com a apoptose também foram determinados. A administração aguda de cisplatina causou toxicidade hepatorrenal em ratos recém-nascidos e adultos. No entanto, o padrão e a intensidade dos danos foram diferentes entre as idades e os tecidos. Os ratos recém-nascidos apresentaram um dano oxidativo mais acentuado em relação aos ratos adultos, caracterizado por um aumento nos níveis de espécies reativas e na proteína carbonila, um menor conteúdo de NPSH e uma maior inibição nas atividades da δ-ALA-D e CAT. Além disso, foi observada uma resposta molecular mais rápida nos níveis proteicos envolvidos com apoptose e resposta ao estresse oxidativo. Em conclusão, os dados deste estudo mostram que o padrão de efeitos tóxicos da cisplatina foi diferente entre as idades e os tecidos dos ratos. Além disso, o presente estudo revelou que os ratos recém-nascidos são mais sensíveis ao tratamento com a cisplatina, pelo menos nas primeiras 24 horas e ainda mostrou que, pelo menos no início, o dano hepático foi maior do que o renal em ratos adultos. Assim, o presente estudo demonstrou que existem diferenças na resposta corporal à exposição aguda da cisplatina entre ratos adultos e recém-nascidos, reforçando a necessidade de mais estudos sobre a resposta da criança à quimioterapia e possíveis estratégias efetivas para minimizar os danos causados pela cisplatina.

Palavras-chave: Cisplatina; nefrotoxicidade; hepatotoxicidade; estresse oxidativo.

ABSTRACT

CISPLATIN INDUCES DIFFERENT HEPATORRENAL PATERN TOXICITY IN NEONATE AND ADULT RATS

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Cisplatin is a widely used chemotherapeutic to treat several types of tumors on both adult and pediatric subjects. Although cisplatin is widely used, its use is associated with serious toxic effects, including nephrotoxicity and hepatotoxicity. Thus, the present study investigated the effects caused by cisplatin acute administration on newborn Wistar rats and the differences in the drug toxicity compared to adult rats. The study was carried out using male and female Wistar rats 10 and 60 day-old (CEUA nº 2699300315). Rats received cisplatin at doses of 5 and 10 mg/kg, by the intraperitoneal (ip.) route, whereas the control group received saline (0.9%, ip.). After 24 h of cisplatin/saline administration, the animals were killed and samples of blood, liver, and kidneys were collected for ex vivo analyses. Markers of renal (urea) and hepatic damage (alanine aminotransferase and aspartate aminotransferase activities) were determined in the serum samples. Parameters of oxidative stress (levels of reactive oxygen species, lipid peroxidation, carbonyl protein, non-protein thiols (NPSH)); activities of antioxidant enzymes (superoxide dismutase, catalase (CAT), glutathione peroxidase, glutathione- S-transferase and glutathione reductase) and activities of sulfhydryl enzymes (δ-aminolevulinate dehydratase (δ-ALA-D) and Na^+, K^+ - ATPase) were determined in samples of liver and kidney of rats. The levels of proteins related to oxidative stress and apoptosis were also determined in samples of liver and kidney of rats at both ages. The cisplatin acute administration caused hepatorenal toxicity in both neonatal and adult rats. However, the pattern and the severity of the damages were different among the ages and tissues. Newborn rats presented greater oxidative metabolic damage when compared to adult rats, characterized by an increase in reactive species and carbonyl protein levels, lower NPSH content, and greater inhibition of δ-ALA-D and CAT activities. In addition, a faster molecular response was found in the protein levels involved with apoptosis and response to oxidative stress. In conclusion, the data from this study show that the pattern of cisplatin toxic effects was different among the ages and tissues of rats. The present study also revealed that newborn rats are more sensitive to treatment with cisplatin for at least the first 24 h and further showed that liver damage, at least in the beginning, was greater than renal damage in adult rats. Thus, the present study demonstrated that there are differences in the body response to acute exposure of cisplatin among adult and newborn rats, reinforcing the need for further studies on children response to chemotherapy and possible effective strategies to minimize the damage caused by cisplatin.

Keywords: Cisplatin; nephrotoxicity; hepatotoxicity; oxidative stress.

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MANUSCRITO

LISTA DE ABREVIATURAS

ALT	Alanina aminotransferase
AST	Aspartato aminotransferase
CAT	Catalase
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa
GSSG	Glutationa oxidada
NADP	Nicotinamida adenina dinucleótideo fosfato
NADPH	Nicotinamida adenina dinucleótideo fosfato reduzida
NPSH	Tiol não-proteico
Nrf2	Fator eritróide nuclear 2 relacionado ao fator 2
SOD	Superóxido dismutase
δ-ALA-D	δ-aminolevulinato desidratase

SUMÁRIO

1 INTRODUÇÃO

1.1 CÂNCER INFANTIL

Câncer é um termo genérico usado para denominar um grande grupo de doenças, um conjunto de mais 100 doenças, que tem como característica principal e comum o crescimento anormal desordenado de células que invadem tecidos e órgãos adjacentes a seu local de origem, e ainda podem espalhar-se para regiões do corpo (EDUCAÇÃO, 2011). O câncer infantil corresponde entre 2% e 3% de todos os tumores malignos, e embora esses números o faça ser considerado raro em comparação com os tumores do adulto, o câncer é a segunda causa de óbito entre crianças de 0 e 14 anos, atrás apenas dos acidentes em países desenvolvidos (CÂNCER, 2008).

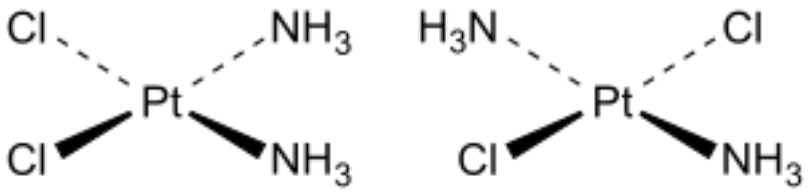
Os tipos de câncer que se desenvolvem em crianças são muitas vezes diferentes daqueles que se desenvolvem em adultos. Na infância, os cânceres são frequentemente resultado de alterações no DNA celular que acontecem no início da vida ou até mesmo na fase fetal (SIEGEL et al., 2016). Em um estudo coordenado pela Agência Internacional para Pesquisa do Câncer (IARC), fora reportado que entre 2001 e 2010 o câncer infantil foi aproxidamente13% mais prevalente que na década de 1980, atingindo uma taxa de incidência anual de 140 milhões de crianças de 0-14 anos em todo mundo (STELIAROVA-FOUCHER et al., 2017). Contudo, as taxas de sobrevivência de crianças com câncer vêm aumentando substancialmente nas últimas décadas (TOMS, 2004).

Embora o aumento das taxas de sobrevivência seja promissor, a baixa especificidade dos tratamentos para o câncer infantil geralmente resulta em efeitos tardios a longo prazo devido ao seu impacto no tecido saudável normal. Assim, sobreviventes de câncer infantil têm um risco aumentado de problemas de saúde e qualidade de vida quando comparados a indivíduos sem histórico de câncer, incluindo maior número e severidade de condições crônicas de saúde, sofrimento psíquico, disfunção neurocognitiva e redução da produtividade (ou seja, incapacidade para o trabalho ou limitação na quantidade ou tipo de trabalho) devido à problemas de saúde (PHILLIPS et al., 2015). Estes resultados têm despertado cada vez mais o interesse sobre a possível toxicidade pós-terapêutica de drogas anticâncer (LANDIER et al., 2015). Um dos quimioterápicos mais utilizados para tratar diversos tipos de tumores pediátricos é a cisplatina (cis-diaminodicloroplatina II (CIS))(RUGGIERO et al., 2013).

1.2 CISPLATINA

A cisplatina foi primeiramente conhecida como cloreto de Peyrone em deferência ao químico italiano Michael Peyrone que a sintetizou em 1844. Sua estrutura foi elucidada posteriormente por Alfred Werner, que propôs existirem dois compostos isômeros um *cis*, como descrito por Peyrone, e um na forma *trans* que havia sido descoberta por Reiset anteriormente (NEVES; VARGAS, 2011). Assim, Werner mostrou que a cisplatina (cis-diaminodicloroplatina (II)) é um composto inorgânico de coordenação com um centro de platina, dois grupos amônia e dois átomos de cloro e possui geometria quadrado planar (Figura 1).

Figura 1. Estrutura dos isômeros *cis* e *trans* da cisplatina



Fonte: Adaptado de NEVES; VARGAS (2011)

Os dados físico-químicos da molécula de cisplatina estão apresentados na Tabela 1.

Tabela 1. Dados físico-químicos da cisplatina

Número CAS	15663-27-1
Fórmula molecular	$Cl_2H_6N_2Pt$
Massa molar	301,1 g/mol
Aparência	pó amarelado, inodoro
Densidade	3,74 g/cm^3
Ponto de fusão	270 ºC
Solubilidade em água	2,53 g/L a 25 ºC

Fonte: Adaptado de DASARI; BERNARD TCHOUNWOU (2014)

Porém foi apenas no século posterior, na década de 60, que sua ação citostática foi descoberta acidentalmente por Rosenberg, quando este estava investigando a ação de um campo elétrico alternado sobre uma cultura de *Escherichia coli* e observou que curiosamente a divisão da célula bacteriana foi inibida, e mesmo não podendo se dividir, as células de *E.*

coli continuavam crescendo na forma de filamentos alongados. Após estes intrigantes achados, Rosenberg continuou investigando e constatou que o agente inibidor era um complexo metálico formado eletroliticamente, produto da reação entre a platina, que se desprendeu dos eletrodos, e o cloreto de amônio, que fazia parte da constituição do meio de crescimento da bactéria (ROSENBERG; VAN CAMP; KRIGAS, 1965).

Intrigado por esse achado, Rosenberg prosseguiu com seus estudos na Universidade do Estado de Michigan onde apurou que apenas o isômero *cis* formado era ativo. Após, a cisplatina e outros complexos de platina foram testados em roedores submetidos a um implante artificial de sarcoma. Dentre os compostos testados, a cisplatina mostrou-se mais eficaz, provocando regressão total do tumor em 36 dias. Sendo assim, na década de 70, começaram os ensaios pré-clínicos em cães e macacos, e após os testes clínicos em pacientes terminais com câncer que não respondiam mais as terapias convencionais. Mesmo havendo um prognostico desfavorável para pacientes nessas condições, a cisplatina obteve resultados animadores, pois cerca de 20% dos pacientes tiveram remissões parciais ou completas. Particularmente, as respostas á cisplatina foram melhores em pacientes com câncer testicular, carcinomas ovarianos, câncer de cabeça e pescoço e também em pacientes com alguns tipos de linfomas. Resultados esses que foram ainda mais satisfatórios quando os estudos chegaram na fase II (DIAS, 1989; KELLAND, 2007).

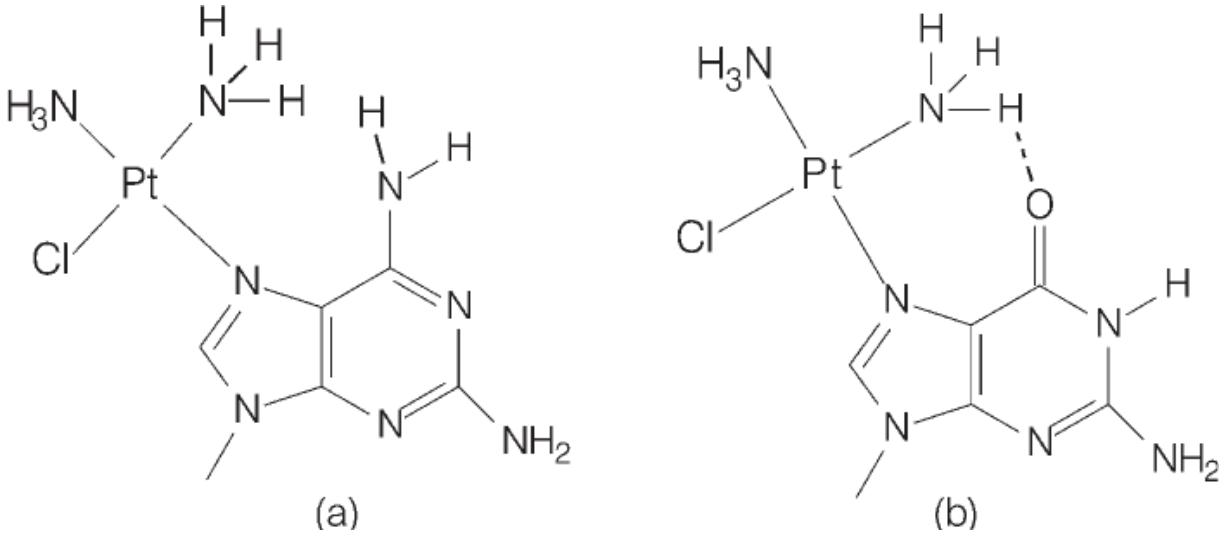
1.3 CISPLATINA COMO UMA DROGA ANTICÂNCER

A cisplatina foi aprovada pelo FDA (*American Food and Drug Administration*) em 1979, desde então, a mesma tem demonstrado potente ação antitumoral, seja como agente isolado ou associado a outros fármacos antitumorais, especialmente nos tumores do testículo e do ovário. Além disso, é também utilizada em regimes de poliquimioterapia para o tratamento de tumores sólidos como: câncer de cabeça e pescoço, câncer prostático e carcinoma de bexiga. Ainda, a cisplatina é efetiva no tratamento de sarcomas, linfomas, câncer de pulmão, câncer de esôfago, neuroblastomas e melanoma (DASARI; BERNARD TCHOUNWOU, 2014; OUN; MOUSSA; WHEATE, 2018).

A cisplatina é administrada pela via intravenosa, na corrente sanguínea liga-se as proteínas plasmáticas, e sua entrada na célula ocorre principalmente através de transportadores de cobre (Ctr1). A molécula torna-se ativa quando seus átomos de cloro são deslocados, de maneira gradual, por moléculas de água, devido a diferença na concentração

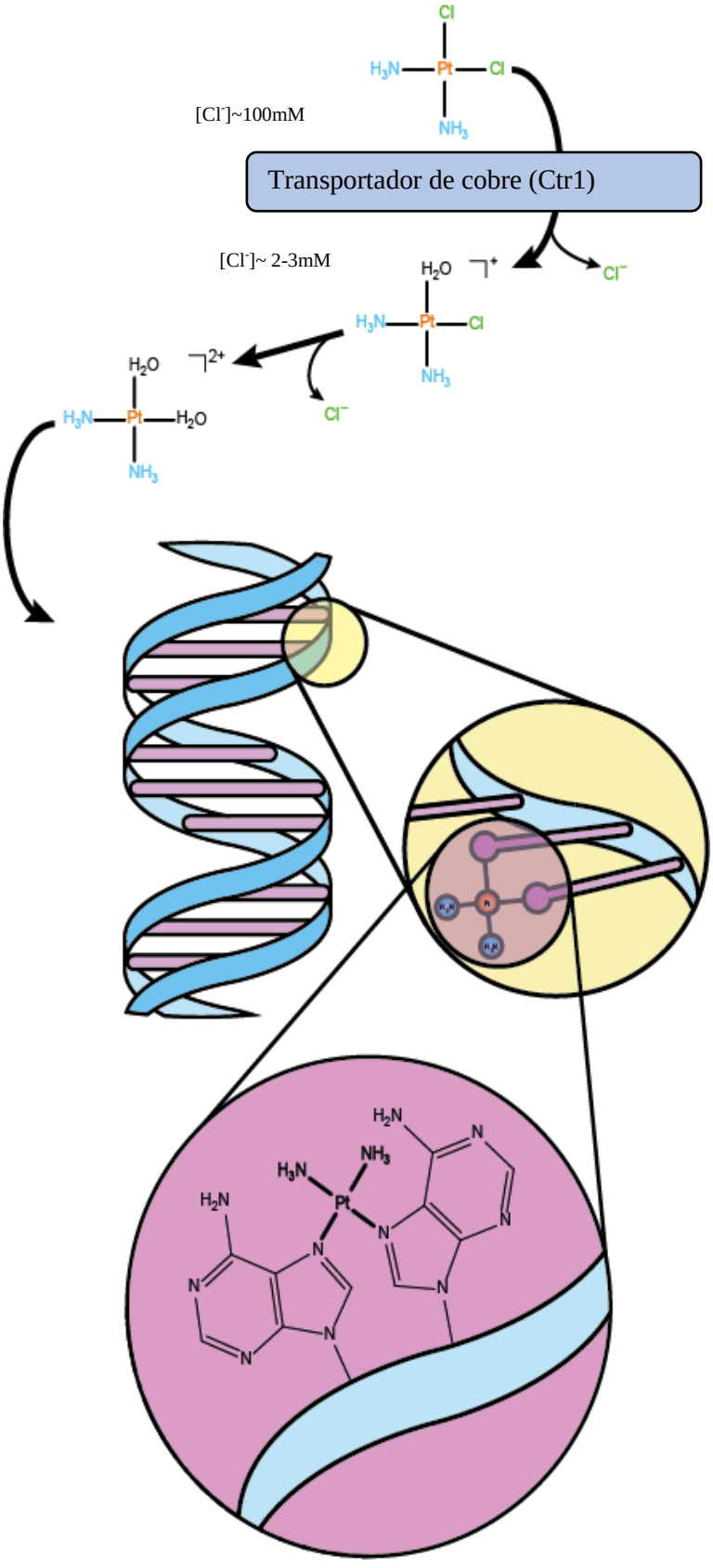
de cloro que cai de 100 mM, no meio extracelular, para 2-3 mM, no meio intracelular. Este produto hidrolisado é um potente eletrófilo que pode reagir com nucleófilos, incluindo grupos sulfidrilicos de proteínas e átomos doadores de nitrogênio de ácidos nucleicos. Evidências indicam que a ação sobre os ácidos nucleicos é o principal mecanismo pelo qual a cisplatina exerce a atividade antitumoral, uma vez que o átomo de platina, da cisplatina, liga-se ao centro reativo N7 nos resíduos de purinas causando modificação no ácido desoxirribonucleico (DNA) das células cancerígenas (Figura 3). Os adutos formados interferem na replicação e transcrição do DNA, inibindo assim a divisão celular e, consequentemente, resultando em morte celular (BAEK et al., 2003; WILMES et al., 2015). A interação dos átomos de platina com o DNA ocorre com maior estabilidade quando a base nitrogenada envolvida é a guanina, pois há possibilidade de formação de ligação de hidrogênio do grupo NH_3 da cisplatina com o oxigênio da guanina, o que não ocorre quando a base nitrogenada envolvida na ligação é a adenina (Figura 2).

Figura 2 Interação da cisplatina com bases nitrogenadas



Fonte: Adaptado de FONTES; DE ALMEIDA; DE ANDRADE NADER (1997)

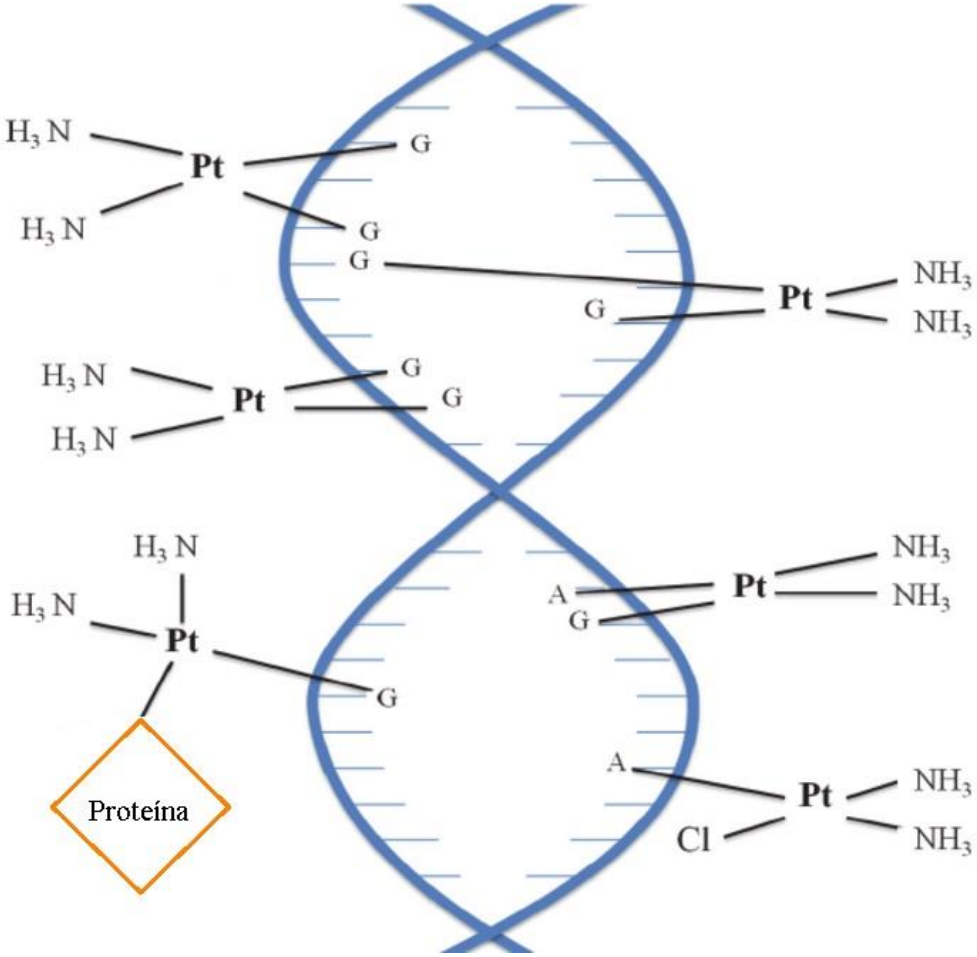
Figura 3. Ligação da cisplatina com o DNA



Fonte: Adaptado de SEBALD; MITCHELL (2017)

A interação da cisplatina com o DNA pode ocorrer de várias formas: formando adutos bifuncionais, quando o átomo de platina se liga as duas posições do DNA, podendo ser na mesma fita (intrafita) ou em fitas diferentes (interfita). Cada átomo de platina pode também fazer apenas uma ligação com o DNA formando um aduto monofuncional. Além disso, a cisplatina pode fazer uma ligação intermolecular, quando o átomo de platina faz uma ligação com o DNA e outra ligação com uma proteína ou aminoácido (Figura 4). Entretanto, as ligações cruzadas 1,2 intrafita com as bases púricas do DNA são as principais responsáveis pela ação citostática da cisplatina, principalmente os adutos 1,2- intrafita d(GpG) e d(ApG) que representam respectivamente cerca de 90% e 10% dos adutos de platina (Pt)-DNA (ACHKAR et al., 2018; PERES; CUNHA JÚNIOR, 2013).

Figura 4. Adutos de cisplatina com o DNA



Fonte: Adaptado de RATANAPHAN (2011)

1.3 TOXICIDADE RELACIONADA A CISPLATINA

Apesar de ser um marco no tratamento contra o câncer e ser amplamente utilizada desde então, a cisplatina também é conhecida por causar inúmeros efeitos adversos graves como: problemas no sistema digestivo, neurotoxicidade, ototoxicidade, cardiotoxicidade, hepatoxicidade e nefrotoxicidade (DASARI; BERNARD TCHOUNWOU, 2014; DILRUBA; KALAYDA, 2016; KATANIĆ et al., 2017; YAO et al., 2007). O efeito tóxico mais frequente causado pela cisplatina é a nefrotoxicidade, que é inclusive um dos maiores fatores limitantes de dose. Estima-se que de 25-35% dos pacientes, após uma única dose do tratamento, apresentam prejuízo na função renal, demonstrado por uma diminuição da taxa de filtração glomerular, aumento da creatinina e ureia, níveis reduzidos de magnésio e potássio séricos (MILLER et al., 2010).

O rim acumula cisplatina em um grau maior do que outros órgãos e é a principal via para a sua excreção. Esse acúmulo desproporcional no tecido renal contribui para a nefrotoxicidade induzida por este fármaco. De fato, a concentração de cisplatina no interior do rim é de cerca de 5 vezes maior que a concentração sérica, sugerindo um acúmulo do fármaco pelas células do parênquima renal (YAO et al., 2007). Além da alta afinidade da cisplatina pelo transportador de cobre (Ctr1), os túbulos proximais renais também expressam um transportador catiônico orgânico (OCT), mais especificamente a isoforma OCT2, que foi identificado como o principal responsável pela entrada de cisplatina na face basolateral dos túbulos proximais renais. Sendo assim o OCT2 é apontado como um transportador crítico e determinante no acúmulo renal da droga, o que ajuda a entender sua toxicidade órgão e célula-especifica (PERES; CUNHA JÚNIOR, 2013).

O fígado é muito importante para a detoxificação de substâncias e é frequentemente, lesado por muitas drogas e xenobióticos e embora menos frequente, a cisplatina pode causar hepatotoxicidade principalmente quando usada em doses elevadas (KATANIĆ et al., 2017). As transaminases são biomarcadores sensíveis e diretamente relacionados com danos celulares, uma vez que são citoplasmáticas e liberados na circulação logo após a lesão tecidual. A hepatoxicidade causada pela cisplatina é evidenciada pela elevação nos níveis de enzimas hepáticas e bilirrubina no soro, além de alterações histopatológicas observadas como a necrose e a degeneração de hepatócitos com infiltração de células inflamatórias ao redor do sistema porta e dilatação sinusoidal (EKINCI AKDEMIR et al., 2017).

Existem muitas evidencias de que o desequilíbrio oxidativo esteja envolvido na

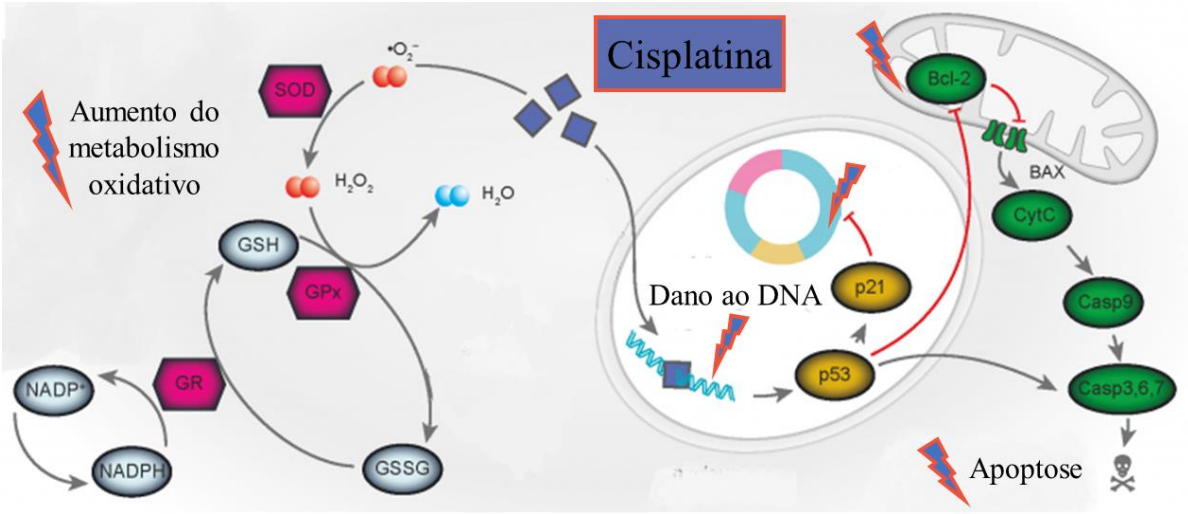
toxicidade induzida pela cisplatina, pois um achado comum nos trabalhos é um aumento na produção de espécies reativas de oxigênio (EROS) e a depleção dos sistemas antioxidantes (BORTOLATTO et al., 2014; OMAR et al., 2016; YOUSEF; HUSSIEN, 2015). Em condições fisiológicas, as células controlam os níveis de espécies reativas de oxigênio, equilibrando a geração de espécies reativas incluindo ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2), e radical hidroxila (OH^-) com a sua eliminação pelos sistemas antioxidantes de defesas enzimáticas: superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e não enzimáticas, principalmente, o tripeptídeo glutationa (GSH). A GSH é uma molécula essencial para a manutenção da integridade celular, pois atua como: cofator de enzimas, substrato para a metabolização de xenobióticos e na manutenção do estado redox celular (NOCTOR et al., 2011).

O aumento do metabolismo oxidativo após o tratamento com a cisplatina resulta em danos significativos à estrutura e funções celulares, incluindo a peroxidação lipídica, nitração de proteínas, inativação enzimática e quebra do DNA. A formação das EROS depende da concentração de cisplatina e da duração da exposição ao fármaco (BROZOVIC et al., 2010). A mitocôndria é o principal alvo do distúrbio oxidativo induzido pela cisplatina, resultando em perda de proteínas sulfidrilicas, inibição da captação de cálcio e redução do potencial de membrana mitocondrial (PERES; CUNHA JÚNIOR, 2013). Outra evidência da participação do estresse oxidativo nos prejuízos celulares causados pela cisplatina é o aumento dos níveis do fator eritróide nuclear 2 relacionado ao fator 2 (Nrf2); uma vez que em condições normais ou na ausência do estresse oxidativo, o Nrf2 é mantido no citoplasma por um conjunto de proteínas que o degradam rapidamente. Sob o estresse oxidativo, o Nrf2 não é degradado, é translocado para o núcleo, onde ele se liga a um promotor de DNA e inicia a transcrição de genes antioxidantes e de suas proteínas relacionadas (OMAR et al., 2016).

A exposição ao estresse oxidativo pode perturbar as funções biológicas regulares, como consequência deste fenômeno ocorre a geração de sinais intracelulares para a ativação tanto de vias apoptóticas, quanto de vias de sobrevivência celular, causando lesão e, consequentemente, morte celular (Figura 5). Um aumento excessivo na produção de EROS pode induzir a morte celular, tanto pela via extrínseca, quanto pela intrínseca da apoptose (OZBEN, 2007). Ambas conduzem à ativação de proteases específicas chamadas de caspases executoras (caspases 3 e 7), resultando em sinais morfológicos característicos de apoptose que incluem formação de bolhas de membrana, retração das células e fragmentação do DNA (SANCHO-MARTÍNEZ et al., 2012). Uma das evidências da apoptose induzida por

cisplatina é o aumento dos níveis da proteína pró-apoptótica, Bax, e a diminuição dos níveis da proteína anti-apoptótica, Bcl_2 (DASARI; BERNARD TCHOUNWOU, 2014; GUMULEC et al., 2014).

Figura 5. Mecanismos de toxicidade relacionados com a cisplatina

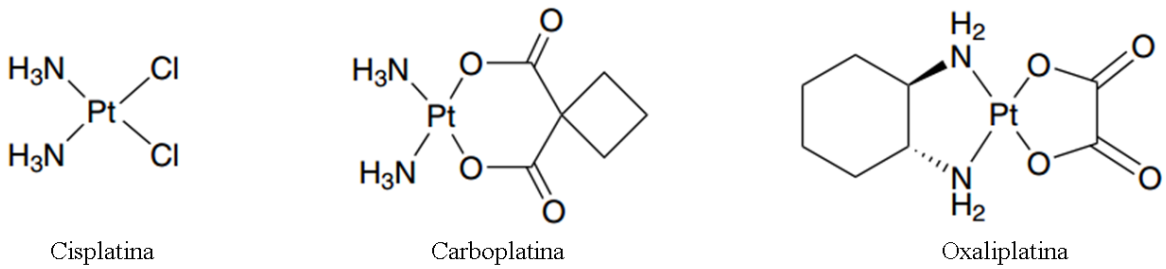


Fonte: Adaptado de GUMULEC et al., (2014)

1.4 OUTROS COMPOSTOS A BASE DE PLATINA

Desde que a cisplatina foi submetida as primeiras analises como possível antineoplásico houve um maior interesse na síntese de compostos a base de platina. Destas drogas, as que obtiveram melhores resultados foram a carboplatina e a oxaliplatina (KELLAND, 2007) (Figura 6).

Figura 6. Drogas a base de platina



Fonte: Adaptado de DILRUBA; KALAYDA (2016)

A carboplatina ou Cis diamino (1,1- ciclobutanodicarboxilato) platina (II), pertencente a segunda geração de drogas quimioterápicas a base de platina, foi aprovada para

comercialização em 1987 e é utilizada para tratar câncer de ovários, pulmão, cabeça e pescoço (RUGGIERO et al., 2013). Em termos de sua estrutura, a carboplatina difere da cisplatina, pois tem um ligante bidentado, o ciclobutanodicarboxilato no lugar dos dois átomos de cloro (Figura 6)(CALVERT et al., 1982). O mecanismo de ação da carboplatina é semelhante ao da cisplatina, porém exibe menor reatividade e cinética de ligação ao DNA mais lenta. Como resultado da reatividade reduzida, os efeitos tóxicos como a neurotoxicidade e ototoxicidade são menos pronunciados, tornando assim a carboplatina adequada para quimioterapia em altas doses (DILRUBA; KALAYDA, 2016). Esta droga é aceita em todo o mundo e quase substituiu a cisplatina em esquemas combinados com paclitaxel para tratamento de câncer de ovário (OZOLS et al., 2003). Mas a dose é limitada por mielossupressão, com trombocitopenia. Além disso a carboplatina tem eficácia limitada contra o câncer de células germinativas testiculares, carcinoma de células escamosas de cabeça e pescoço e câncer de bexiga. O que faz com que a cisplatina ainda permaneça sendo a droga de escolha para o tratamento dessas doenças (DILRUBA; KALAYDA, 2016).

Aprovado para uso pelo FDA em 2002, a oxaliplatina (trans-1,2-diaminocicloexanooxalato) platina (II) é um composto de platina de terceira geração e tem atividade antitumoral de amplo espectro contra o neuroblastoma, carcinoma ovariano, câncer pulmão não-pequenas células, carcinoma de cólon e bexiga, além de alguns tipos de câncer de mama (RUGGIERO et al., 2013). A estrutura da oxaliplatina inclui um átomo de platina central circundado por um grupo 1,2-diaminociclohexano um ligante de oxalato bidentado (Figura 6). O efeito citotóxico da droga também está relacionado com a formação de adutos de platina com o DNA. Ao contrário de outros compostos de platina, o seu perfil de toxicidade é aceitável, com uma incidência de ototoxicidade inferior a 1%, toxicidade renal inferior a 3%. Entretanto, há relatos de neurotoxicidade, especificamente, em relação a nervos sensoriais, o que dificulta a continuidade do tratamento (DILRUBA; KALAYDA, 2016).

Apesar de passados 40 anos desde sua aprovação para comercialização, a cisplatina é um dos agentes anticancerígenos mais eficazes usado no tratamento de tumores sólidos, sendo amplamente utilizada para a cura de diferentes tipos de neoplasias, incluindo as pediátricas como o neuroblastoma, o osteossarcoma e alguns tumores cerebrais (SKINNER et al., 2009). Devido à grande e constante demanda de uso da cisplatina, principalmente em pacientes pediátricos e a crescente preocupação com seus efeitos tóxicos, durante e após o tratamento, é importante saber quais são os efeitos tóxicos desse medicamento quando usado no início da vida. A fim de prevenir as consequências que esse fármaco pode causar no desenvolvimento

do indivíduo, principalmente, nos órgãos em que a cisplatina tem contato nas primeiras horas após a administração, o fígado onde sofre metabolização e o rim onde é eliminada.

2 OBJETIVOS

2.1 OBJETIVOS GERAIS

Avaliar se a administração aguda de cisplatina em ratos recém-nascidos causa danos hepáticos e renais e comparar esses danos com os causados pela administração aguda de cisplatina em ratos adultos.

2.2 OBJETIVOS ESPECÍFICOS

Investigar os efeitos da administração de cisplatina em ratos Wistar com 10 dias e a diferença na resposta a cisplatina entre as idades, quando comparados com ratos adultos sobre:

- parâmetros de dano renal (níveis de creatinina, ureia) e hepático (atividade das enzimas alanina aminotransferase (ALT), aspartato aminotransferase (AST)), no plasma;
- os níveis de EROS, peroxidação lipídica, cabonilação de proteínas e NPSH no fígado e rim;
- as atividades das enzimas antioxidantes SOD, CAT, GPx, GST e GR no fígado e rim;
- as atividades das enzimas sulfidrílicas δ-aminolevulinato desidratase (δ-ALA-D) no fígado e no rim e Na^+, K^+-ATPase no rim, como marcadores de exposição;
- os níveis das proteínas relacionadas com o estresse oxidativo e apoptose.

3 DESENVOLVIMENTO

O desenvolvimento dessa dissertação está apresentado na forma de um manuscrito. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se descritos no próprio manuscrito, que está estruturado de acordo com as normas da revista ao qual foi submetido.

Pattern differences between newborn and adult rats in cisplatin-induced hepatorenal toxicity

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Abstract

Although cisplatin (CIS) has been associated with serious adverse effects, such as hepatotoxicity and nephrotoxicity in adult rats, there is few reports on its use in newborn rats. The aim of this study was to evaluate acute toxic effects of CIS in newborn rats. Adult and newborn Wistar rats received CIS by the intraperitoneal route, at the dose of 5 or 10 mg/kg. After 24 h of treatment, blood, kidney, and liver were excised from the animals and parameters of renal and hepatic functions, oxidative stress markers were determined. Acute administration of CIS caused an increase of aspartate aminotransferase activity and urea levels, suggesting hepatorenal toxicity in newborn and adult rats. However, the pattern and intensity of damage was different between ages and tissues. Newborn rats showed more pronouncedly oxidative stress damage, characterized by an increase in reactive species and protein carbonyl levels, lower non-protein thiol content and highest inhibition in δ-aminolevulenic acid dehydratase and catalase activities. Besides that, it was observed a faster molecular response in protein levels involved with apoptosis and oxidative stress response; whereas in the beginning the damage was less severe in the kidney than in the liver of adult rats. Thus, the present study shows that there are body response differences between adult and newborn rats to CIS acute exposure being that newborn rats are more susceptible than adults.

Keywords: cisplatin; toxicity; hepatorenal; newborn; adult.

1 Introduction

Childhood cancer was approximately 13% more prevalent between 2001 and 2010 than in the 1980s, reaching an annual incidence rate of 140 million children aged 0-14 years worldwide, being considered the major cause of death by disease in this population [1]. However, along with this increase in cases there was also a substantial growth in five-year survival rate of children and adolescents with cancer. This improvement in survival rates is due to treatment progress in more equitable access to timely and effective treatment programmes [2, 3].

Cisplatin (CIS) is one of the most used chemotherapeutic agents used in the treatment of adult and pediatric neoplasms. CIS is an alkylating agent containing platinum that is used alone or in combination with other antineoplastic agents to treat a broad spectrum of childhood malignancies, such as osteosarcomas, neuroblastomas, germ cell tumors and relapse or refractory lymphomas [4, 5]. CIS becomes active inside the cell, once in cytoplasm the chloride atoms of CIS are replaced by water molecules, leading to a potent electrophile that can react with intracellular nucleophiles, including sulfhydryl groups in proteins and nitrogen donor atoms of nucleic acids. Thus, it can lead to the formation of adduct with DNA resulting to the blocking of cell division and culminating in the process of cell death [6]. Despite its widespread use, CIS is associated with a number of serious adverse effects such as: nephrotoxicity, hepatotoxicity, neurotoxicity, and ototoxicity [7-9].It is widely known that the body has a prolonged retention of platinum because twenty years after the end of treatment it is still possible to find platinum in the body [10].

Some studies report the role of oxidative stress in treatment-related adverse effects, because many of the damages caused by CIS are the result of free radical formation, lipid peroxidation of cell membranes as well as the reduction of antioxidant enzyme activities and depletion of antioxidant substrates, mainly the tripeptide glutathione (GSH) [11-14]. Among the main toxic effects of CIS are hepatotoxicity and nephrotoxicity, which are closely related to the destabilization of the redox balance caused by the antineoplastic [15, 16]. In addition, previous studies showed that treatment with CIS causes structural changes in the liver and kidney tissues, which may lead to impairment of their function [17, 18]. A recent study reported by Akdemir et al [19] showed that acute administration of CIS at the dose of 10 mg/kg in adult Wistar rats caused damage to the liver and kidney, such as severe degeneration and tubular necrosis in kidneys and sinusoidal dilatation, degeneration and vascular

congestion in hepatocytes, in addition to increased production of reactive species and loss of antioxidant system in both tissues.

Because children and adults are affected by different types of cancer and respond differently to chemotherapeutic treatment [20] and that CIS causes acute renal and hepatic damage in Wistar adult rats [19], the aim of this study was to evaluate the acute toxic effects of CIS in newborn rats. Furthermore, a few studies reveal the toxic effects of CIS in rats at the developmental stage.

2 Materials and methods

2.1 Animals

Male and female newborn rats (post-natal day 10) and adult (post-natal day 60) Wistar rats were obtained from our breeding colony. The animals were kept under controlled temperature (22 ± 2 ºC) and humidity (45% to 65%) conditions on a 12-h light/12-h dark cycle, with lights turned on at 7.00 a.m. Rats were housed in polycarbonate cages, the diet consisted of commercial feed (GUABI, RS, Brazil) and fresh water *ad libitum*. The experiments that are described in this study were carried out in accordance with the rules of Committee on Care and Use of Experimental Animal Resources from the Federal University of Santa Maria-RS - Brazil and registered under the number (#2699300315). The procedures in this study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Drugs

Cisplatin (*cis*-diamminedichloridoplatinum II, C-Platin®; Blau, SP, Brazil) was obtained through the donation of the University Hospital of Santa Maria, Brazil (HUSM). A cocktail of protease inhibitor and the bicinchoninic acid assay (BCA) were obtained from Sigma (Sigma-Aldrich Company, St. Louis, Missouri, United States). A prestained protein standard was purchased from Bio-Rad (Bio-Rad, São Paulo, Brazil). All other chemicals used in this study were of analytical grade and obtained from common commercial sources.

2.3 Experimental Design

Firstly, rats were randomly divided into two groups according to their age: newborn or adult group. After, animals were regrouped for drug treatments in three subgroups: Control, CIS 5, and CIS 10, totalizing six experimental groups (three groups for newborn and three groups for adult rats). Rats in the control group received a 0.9% saline solution by the intraperitoneal (i.p) route, whereas rats in CIS groups received CIS (i.p.) at the dose of 5 or 10 mg/kg. After 24 h of treatment, animals were anesthetized with sodium pentobarbital at the dose of 150 mg/kg (i.p.), and they were killed by cardiac exsanguination. The liver and kidney samples were rapidly dissected, weighed, and placed on ice. Tissue samples were used to determine oxidative stress markers and to detect the levels of some proteins by western blot analysis.

2.4 Hepatic and renal function markers

Blood samples were centrifuged at 2000×g for 10 min to obtain the plasma fraction. Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined as a parameter of hepatic function and the urea levels for the functionality of kidneys. These parameters were determined by commercial kits (Labtest Diagnostica, MG, Brazil). The results of AST and ALT activities were expressed as U/dL, whereas urea levels were expressed as mg/dL.

2.5 Tissue preparation

Samples of liver and kidney were homogenized (1:10 w/v) in 50 mM Tris–HCl at pH 7.4 and centrifuged at 2000× g for 10 min at 4°C. The resulting supernatants (S1) or the homogenates without centrifugation were used to biochemical analysis. Protein concentration in S1 was determined according to the method of Bradford [21], using bovine serum albumin (1 mg/mL) as a standard. The color was measured spectrophotometrically at 595 nm.

2.6 Oxidative damage markers

2.6.1 Protein carbonyl content

Reaction of carbonyl proteins with dinitrophenylhydrazine (DNPH) to form dinitrophenylhydrazone is a method to measure carbonyl content [22].Homogenate was

diluted with Tris-HCl buffer, pH 7.4 in a ratio of 1:10. Aliquots of 1 mL of these dilutions were placed in tubes with 200 μL of 10 mM DNPH in 2M HCl or only 200 μL of 2M HCl (blank). Afterwards all tubes were incubated for 1 h at room temperature in the dark and shaken using a vortex mixer every 15 min. After that, 0.5 mL of denaturation buffer (sodium phosphate buffer, pH 6.8, containing 3%sodium dodecyl sulfate (SDS)), 1.5 mL of ethanol, and 1.5 mL of hexane were added to all tubes. Immediately tubes were vortexed for 40s and centrifuged for 15 min at 2400 ×g. Supernatants were discarded and obtained pellet was separated, washed twice with 1 mL of a mixture of ethanol: ethyl acetate (1: 1, v/v), and dried at room temperature for 2 min. The pellet was immediately dissolved in 1mL of denaturation buffer. Absorbance was measured at 370 nm. Results are expressed as nmol carbonyl/mg protein.

2.6.2 Reactive species (RS)

RS levels were measured as reactive species to 2', 7'-dichlorofluorescein diacetate (DCHF-DA). DCHF-DA is used as a fluorescent probe to measure RS levels, as it is easily oxidized to fluorescent dichlorofluorescein (DCF) [23]. The oxidation of DCHF-DA to DCF was determined at 488 nm for excitation and 525 nm for emission. An aliquot of 1mM DCHF-DA in ethanol was added to a mixture containing 10 μL of S1 and 3 mL of 10 mM Tris-HCl pH 7.4 and incubated for 1h at 37 °C, protected from light. DCF fluorescence intensity was expressed as fluorescence intensity (arbitrary units)/mg protein.

2.6.3 Malondialdehyde (MDA) levels

MDA levels have been recognized as an important lipid peroxidation indicator [24]. An aliquot of 75 μL of S1 was added in 3M NaOH and incubated at 60°C for 30 min. Thereafter, 6% H_3PO_4 and 0.8% thiobarbituric acid (TBA) were added to the system and the mixture was incubated again at 90 °C for 2 h. Following, a volume of 10% (SDS) and n-butanol were added to extract the TBA-MDA adduct, which was analyzed on Shimadzu® HPLC equipment. The analytical column used was a Phenomenex® ODS-2C18 reverse phase (250 mm 4.6 mm, 5 μm, 100 Å, Allcrom, SP, Brazil) and the mobile phase was ultrapure water and methanol (50:50; v/v). HPLC analysis were performed under isocratic conditions at a flow rate of 0.6 mL/min and UV detector set at 532 nm with injection of 20 μL sample volume. The results were expressed as nmol MDA/mg protein.

2.7 Non-enzymatic antioxidant defense

2.7.1 Non-protein thiol (NPSH) content

The levels of NPSH were determined according to the method described by Ellman [25]. S1 was mixed (1:1) with 10% trichloroacetic acid (TCA). After centrifugation (2400 ×g for 10 min), the protein pellet was discarded and the free SH-groups were determined in the clear supernatant. An aliquot of the supernatant was added in 1M potassium phosphate buffer pH 7.4 and 10 mM DTNB (5,5'-dithiobis-2-nitrobenzoic acid). The color reaction was measured at 412 nm and NPSH levels were expressed as nmol of NPSH/g tissue.

2.8 Sulfhydryl enzymes sensitive to oxidative stress

2.8.1 δ- aminolevulinic acid dehydratase activity

δ-ALA-D activity was measured through the rate of product (porphobilinogen (PBG)) formation, according to the method described by Sassa [26]. The S1 (200 μL) was preincubated for 10 min at 37 °C with 1M potassium phosphate buffer pH 6.8. The enzymatic reaction was initiated by the addition of the substrate (δ-ALA) and incubated for 1h at 37 °C. The reaction was stopped by the addition of 10% TCA solution with 10 mM $MgCl_2$. The reaction product was measured at 555 nm using Ehrlich's modified reagent and values were expressed as nmol PBG/mg protein/h.

2.8.2 Na^+, K^+-ATPase activity

The Na^+, K^+-ATPase activity was measured only in kidney samples. The reaction mixture for Na^+, K^+-ATPase activity assay contained: 6 mM MgCl, 100 mM NaCl, 20 mM KCl, 40 mM Tris–HCl, pH 7.4, and 50 μL of S1 in a final volume of 500 μL. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow [27] and expressed as nmol de Pi/mg protein/min.

2.9 Antioxidant enzymes

2.9.1 Superoxide dismutase (SOD) activity

SOD activity was assayed spectrophotometrically as described by Misra and Fridovich [28]. This method is based on the capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome. Briefly, S1 was diluted 1:10 (v/v) for determination of SOD activity in test day. Aliquots were added in a 0.05 M Na_2CO_3 buffer pH 10.2. Enzymatic reaction was started by adding of the epinephrine. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50 % at 26°C. The enzymatic activity was expressed as U / mg protein.

2.9.2 Catalase (CAT) activity

CAT activity was spectrophotometrically assayed by monitoring the H_2O_2 consumption at 240 nm according to Aebi [29]. Aliquots of S1 were added in 50 mM potassium phosphate buffer pH 7.0 and the enzymatic reaction was initiated by adding H_2O_2. The enzymatic activity was expressed in Units (1U decomposes 1 μmol H_2O_2/min at pH 7 at 25°C)/ mg protein.

2.9.3 Glutathione Peroxidase (GPx) activity

GPx activity was measured spectrophotometrically by the dismutation of H_2O_2 at 340 nm as described by Wendel [30]. S1 was added in the glutathione (GSH)/NADPH/glutathione reductase *(*GR) system and the enzymatic reaction was initiated by the addition of H_2O_2. In this assay, the enzymatic activity is indirectly measured by NADPH decay. H_2O_2 is reduced and generates GSSG from GSH. GSSG is regenerated back to GSH by the GR present in the analysis medium at the expense of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.9.4 Glutathione Reductase (GR) activity

GR activity in S1 was determined through consuming NADPH to reduce oxidized glutathione (GSSG), which was measured at 340 nm as described by Carlberg and Mannervik [31]. GR activity is proportional to the decay of NADPH. The enzymatic activity was expressed as nmol of NADPH/min/mg of protein.

2.9.5 Glutathione S transferase (GST) activity

GST activity was measured spectrophotometrically through conjugating GSH to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm as described by Habig, Pabst and Jakoby [32]. An aliquot of 50 μL of S1 was added to 0.1M potassium phosphate buffer pH 7.4; 100 mM CDNB, and 100 mM GSH to be used as the substrate. The enzymatic activity was expressed as nmol of conjugated CDNB/min/mg protein.

2.10 Western blot assay

Liver and kidney samples were homogenized in HEPES-sucrose buffer pH 7.4 containing a cocktail of protease inhibitor. The protein content was determined using the BCA kit assay. The homogenates were diluted to a final protein concentration of 2 μg / μL in a buffer (consisting essentially of 500 mM Tris / HCl pH 6.8, glycerol, 10% SDS, 2-β-mercaptoethanol and 2% bromophenol blue used as a marker of the electrophoresis process). The samples (20 μg protein / well) as a marker protein were separated on an SDS-polyacrylamide gel by electrophoresis. The proteins were transferred to a nitrocellulose membrane (0.45 μm, Bio-Rad) using the Transfer-Blot® Turbo ™ transfer system (1.0 mA, 45min, Bio-Rad). After blocking with 3% bovine serum albumin (BSA) solution for 1 h, the blots were incubated overnight at 4 °C with mouse anti-Bax (1:1000), rabbit anti-Nrf2 (1:1000), goat anti-Keap1(1:1000), goat anti-phospho-PKCα (Ser 657) (1:1000), rabbit anti-PKCα (1:5000) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit anti-Bcl2 (1:3000), rabbit anti- cleaved caspase-3 (Asp175) (1:1000), rabbit anti-phospho-Akt (Ser473) (1:1000), rabbit anti-Akt (1:1000) (Cell Signaling Technology, Beverly, MA, USA). Rabbit anti-GAPDH (1:1000, Cell Signaling) was stained as a constitutive protein. After incubation with the primary antibodies, the membranes were washed and also incubated with the respective peroxidase-conjugated secondary antibodies for 1 hour at 2-8°C. A chemiluminescence kit (Amersham, São Paulo/Brazil) was used for protein detection and the signals were captured with Amersham Imager 600 (GE healthcare life sciences). Optical density (O.D.) was performed using Image J (NIH, Bethesda, MD, USA) software for Windows. Each value was derived from the ratio between arbitrary units obtained by the protein band and the respective GAPDH band. The results were expressed as percentage of control.

2.11 Statistical Analysis

All experimental data were analyzed by the One-way ANOVA, followed by the Newman-Keuls test, excepting those obtained in the Western blot assay that were analyzed using unpaired Student's t test. Differences among groups were considered statistically significant when probability values were less than 0.05 ($P<0.05$). Descriptive statistics data were expressed as the mean(s) ± SEM. All analyses were performed using the GraphPad software (GraphPad software, San Diego, CA, USA).

3 Results

3.3 CIS affects parameters of hepatic and renal function

The results illustrated in table 1 show the activities of AST and ALT, and urea levels in plasma of rats. The administration of CIS at doses of 5 and 10 mg/Kg increased the activity of AST in newborn (One-way ANOVA [$F_{(2, 15)} = 15.85$, $P<0.001$]) and adult rats (One-way ANOVA [$F_{(2, 15)} = 8.019$, $P <0.005$]). According to the One-way ANOVA analysis, ALT activity was not modified in the experimental groups in both ages. Regarding to the urea levels the statistical analysis showed a significant difference among the experimental groups. The post hoc evaluation demonstrated that CIS administered at doses of 5 and 10 mg/Kg increased the plasma levels of urea in newborn (One-way ANOVA [$F_{(2, 15)} = 30.63$, $P<0.0001$]) and in adult rats (One-way ANOVA $F_{(2, 15)} = 4.187$, $P<0.05$]).

Table 1: Effects of CIS administration on hepatic and renal function markers in newborn and adult rats.

	Newborn			Adult		
	Control	CIS 5	CIS 10	Control	CIS 5	CIS 10
AST	82.00±7.04	111.80±12.28$^{\#}$	236.00±32.60$^{\#\&}$	91.60±4.60	92.00±6.39	117.80±4.74$^{\#\&}$
ALT	32.50±5.69	35.40±4.87	36.50±6.74	54.50±3.53	51.40±5.42	48.00±3.03
UREA	57.45±5.78	86.09±4.63$^{\#}$	120.40±6.51$^{\#\&}$	38.41±2.16	56.65±5.95$^{\#}$	60.81±7.84$^{\#}$

Values are reported as means ± S.E.M. of six animals per group. Data were analyzed using a One-way analysis of variance (ANOVA) followed by the Newman - Keuls test. AST and ALT activities are expressed as U/dL. Levels of urea are expressed as mg/dL. # denotes the significance levels when compared to the control group. & denotes the significance levels when compared to the CIS 5 mg/Kg group.

3.4 CIS affects oxidative damage markers

CIS administered at the dose of 10 mg/Kg increased the protein carbonyl levels in the liver (One-way ANOVA [$F_{(2, 15)} = 9.883$, $P<0.005$]) and kidney (One-way ANOVA [$F_{(2, 15)} = 5,764$, $P<0.05$]) of newborn rats (Fig. 1A). The administration of CIS to adult rats did not change protein carbonyl levels.

The One-way ANOVA of RS levels showed a significant difference among the experimental groups (Fig. 1B). Newborn rats administered with CIS at the dose of 10 mg/kg showed an increase in RS levels in the liver (One-way ANOVA [$F_{(2, 15)} = 6.426$, $P<0.05$]), these levels were increased in kidney at both doses of CIS (One-way ANOVA [$F_{(2, 15)} = 4.810$, $P<0.05$]). In adult rats, both doses of CIS increased RS levels in the liver (One-way ANOVA [$F_{(2, 15)} = 7.756$, $P<0.05$]), whereas in the kidney CIS only at the dose of 10 mg/Kg increased RS levels (One-way ANOVA [$F_{(2, 15)} = 3.784$, $P<0.05$.]). All experimental groups of newborn and adult rats showed similar MDA levels (Table 2).

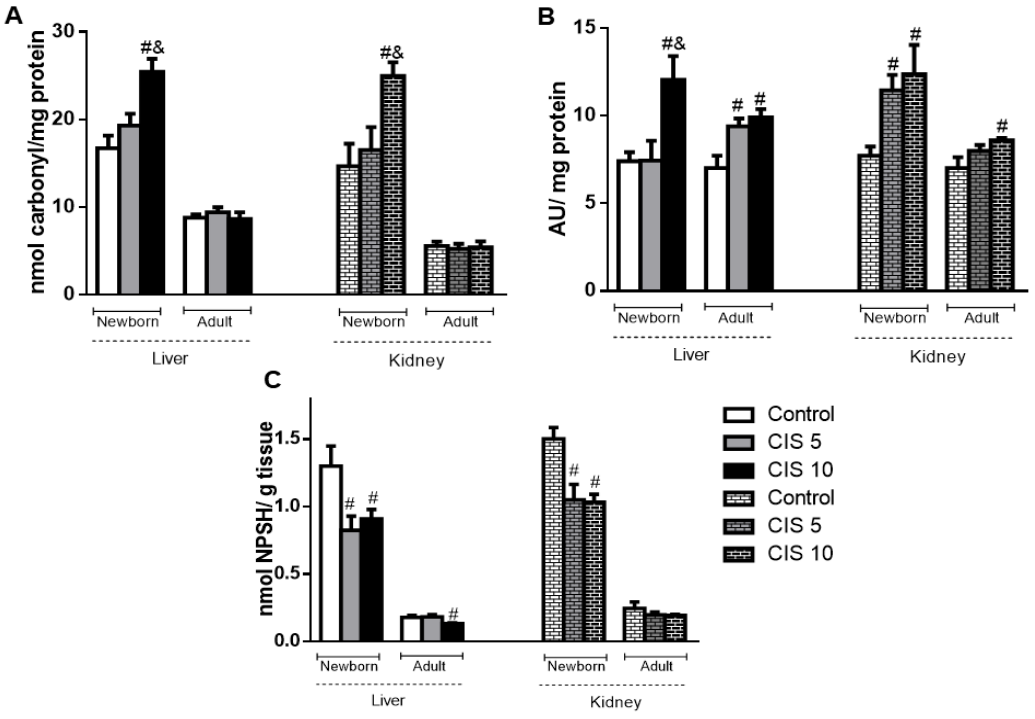

Figure 1. Effects of CIS administration on protein carbonyl levels (A), RS levels (B) and NPSH content (C) in liver and kidney of newborn and adult rats. Values are expressed as the mean ± SEM (n = 6 rats/group). Data were analyzed by one-way ANOVA followed by the Newman - Keul's test for post hoc comparison when appropriate. # denotes the significance levels when compared to the control group. & denotes the significance levels when compared to the CIS 5 mg/Kg group.

3.5 CIS affects a non-enzymatic antioxidant defense

CIS administered at doses of 5 and 10 mg/kg decreased NPSH levels in the liver (One-way ANOVA [$F_{(2, 15)} = 5.086$, $P<0.05$]) and kidney (One-way ANOVA [$F_{(2, 15)} = 9.018$, $P<0.005$]) of newborn rats. All experimental groups of adult rats showed similar NPSH levels in the liver and kidney (Fig.1C).

3.6 CIS inhibits sulfhydryl enzyme activities

The administration of CIS at the dose of 10 mg/kg inhibited the hepatic δ-ALA-D activity in newborn (One-way ANOVA [$F_{(2, 15)} = 5.770$, $P<0.05$]) and adult rats (One-way ANOVA [$F_{(2, 15)} = 7.705$, $P<0.01$]). In kidney, both doses of CIS decreased the δ-ALA-D activity in both ages (One-way ANOVA [$F_{(2, 15)} = 5.373$, $P<0.05$], [$F_{(2, 15)} = 4.877$, $P<0.05$]) (Fig. 2A).

The results illustrated in figure 2B show the Na^+, K^+-ATPase activity in kidney. The administration of CIS at a dose of 10 mg/kg decreased the enzyme activity in newborn (One-way ANOVA [$F_{(2, 15)} = 5.486$, $P<0.05$]) and adult rats (One-way ANOVA [$F_{(2, 15)} = 4.041$, $P<0.05$]).

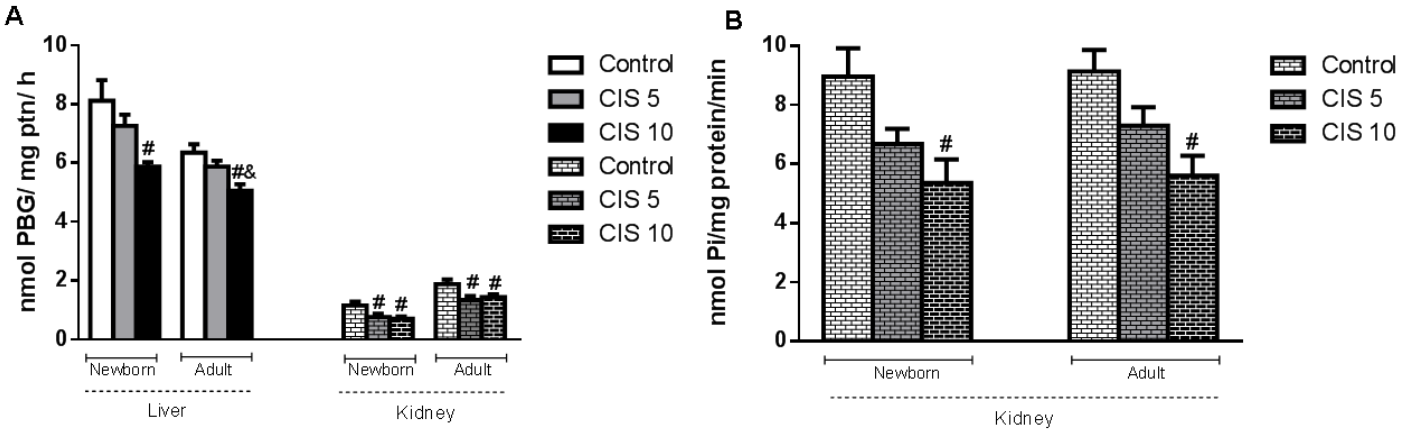

Figure 2. Effects of CIS administration on δ-ALA-D activity (A) in liver and kidney of newborn and adult rats. The Na^+, K^+-ATPase activity (B) in kidney of newborn and adult rats. Values are expressed as the mean ± SEM (n = 6 rats/group). Data were analyzed by one-way ANOVA followed by the Newman - Keul's test for post hoc comparison when appropriate. # denotes the significance levels when compared to the control group. & denotes the significance levels when compared to the CIS 5 mg/Kg group.

3.7 CIS affects enzymatic antioxidant defenses

As shown in table 2, the hepatic CAT activity was reduced by CIS administered at doses of 5 and 10 mg/kg to newborn (One-way ANOVA [$F_{(2, 15)} = 12.58$, $P<0.001$]) and adult rats (One-way [$F_{(2, 15)} = 10.65$, $P<0.005$]). Similar pattern was observed in the kidney of adult rats (One-way [$F_{(2, 15)} = 6.994$, $P<0.01$]) but in newborn rats only the dose of 10 mg/kg decreased the CAT activity (One-way [$F_{(2, 15)} = 22.96$, $P<0.0001$]). No change in the SOD activity was observed after CIS administration in all experimental groups at both ages.

Table 2 also shows the enzymatic activities of GPx, GR and GST. The highest dose of CIS increased the GPx activity in the liver (One-way [$F_{(2, 15)} = 3.783$, $P<0.05$]) and kidney (One-way [$F_{(2, 15)} = 3.963$, $P<0.05$]) of adult rats. The GPx activity was not altered in all experimental groups of newborn rats. The administration of CIS at the dose of 10 mg/kg increased the GR activity in the liver (One-way [$F_{(2, 15)} = 6.185$, $P<0.05$]) and kidney (One-way [$F_{(2, 15)} = 5.254$, $P<0.05$]) of newborn animals. In adult rats, GR activity was not modified in the experimental groups. The hepatic GST activity was decreased in adult rats after administration of CIS at the dose of 10 mg/kg (One-way [$F_{(2, 15)} = 5.278$, $P<0.05$]). In the other experimental groups, no changes were observed in the GST activity.

Table 2: Effects of CIS administration on hepatic and renal oxidative stress parameters of newborn and adult rats.

	Newborn			Adult		
	Control	CIS 5	CIS 10	Control	CIS 5	CIS 10
			Liver			
MDA	0.54±0.89	0.66±0.07	0.41±0.03	0.10±0.01	0.11±0.01	0.116±0.01
SOD	89.70±3.33	89.29±6.76	92.63±4.68	100.40±4.72	97.01±2.01	97.19±3.54
CAT	9.46±0.87	5.05±0.67$^{\#}$	5.16±0.53$^{\#}$	19.38±1.70	13.22±1.37$^{\#}$	11.22±0.52$^{\#}$
GPx	1411.00±25.06	1374.00±44.42	1382.00±29.61	977.60±52.25	1118.00±28.78	1185.00±72.72$^{\#}$
GR	821.40±22.37	826.50±23.76	920.50±21.12$^{\#\&}$	623.90±43.02	700.40±28.12	668.80±13.81
GST	1144.00±17.43	987.70±45.97	1026.00±57.81	3753.00±329.10	3094.00±189.90	2760.00±38.17$^{\#}$
			Kidney			
MDA	0.17±0.01	0.19±0.02	0.21±0.02	0.11±0.01	0.09±0.01	0.11±0.01
SOD	62.94±3.90	66.52±10.20	67.66±6.94	27.75±2.27	24.59±0.90	22.21±2.14
CAT	2.17±0.09	1.84±0.15	1.06±0.09$^{\#\&}$	9.99±0.70	7.64±0.27$^{\#\&}$	7.99±0.33$^{\#\&}$
GPx	635.30±12.37	632.30±52.36	662.50±15.43	332.10±8.92	314.90±9.61	348.50± 6.46$^{\#}$
GR	91.95±7.90	114.60±12.16	163.20±23.36$^{\#\&}$	150.20±5.78	139.40±5.01	125.30±6.24$^{\#}$
GST	303.20±30.47	284.30±26.01	293.90±22.27	104.60±5.97	100.20±2.78	100.30±3.57

Values are reported as means ± S.E.M. of six animals per group. Data were analyzed using a One-way analysis of variance (ANOVA) followed by the Newman - Keuls test. MDA levels are expressed as nmol MDA / mg protein. The enzymatic activity of CAT and SOD are expressed as U/mg protein. GPx and GR activity are expressed as nmol of NADPH/min/mg protein and GST activity is expressed as nmol of conjugated CDNB/min/mg protein. # denotes the significance levels when compared to the control group. & denotes the significance levels when compared to the CIS 5 mg/Kg group.

3.8 CIS decreases Nrf2/keap1 ratio in newborn rats

An unpaired Student's t test revealed that the highest dose of CIS decreased the Nrf2/Keap1 ratio in the liver ($P<0.05$) and kidney ($P<0.005$) of newborn rats. The Nrf2/keap1 ratio was not altered by CIS in adult rats (Fig. 3A).

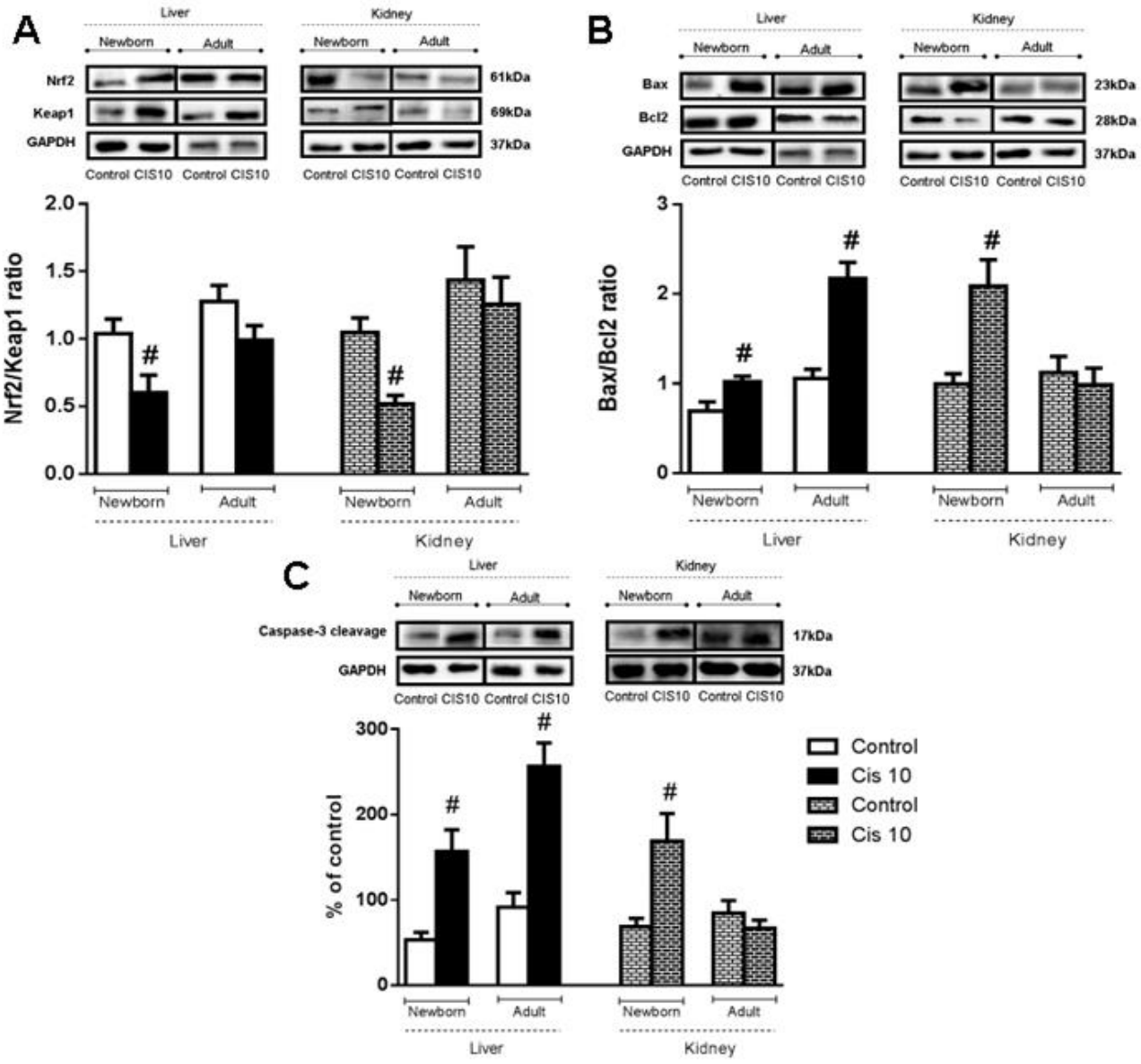

Figure 3. Effects of CIS administration on the Nrf2/Keap1 ratio (A), Bax/Bcl2 ratio (B) and caspase-3 cleavage levels (C) in liver and kidney of newborn and adult rats. Values are expressed as the mean ± SEM (n = 5 rats/group). Data were analyzed by unpaired Student's t test. # denotes the significance levels when compared to the control group. Photographs are representation of qualitative Western blotting analysis.

3.9 CIS affects proteins involved in apoptosis

Figure 3B shows the increase in Bax/Bcl2 ratio in the liver ($P<0.05$) and kidney ($P<0.01$) of newborn rats administered with CIS. Similar pattern for the Bax/Bcl2 ratio was found in the liver ($P<0.001$) but not in the kidney of adult rats.

Unpaired Student's t test revealed that the highest dose of CIS induced an increase in caspase-3 cleavage levels in the liver ($P<0.005$) and kidney ($P<0.05$) of newborn rats. In adult rats, an increase of caspase-3 cleavage levels was found in the liver ($P<0.001$) but no change was found in the kidney (Fig. 3C).

3.10 CIS affects phosphorylation of Akt

CIS-treated rats showed an increase of p-Akt/Akt ratio in the liver ($P<0.05$) and kidney ($P <0.0001$) of newborn rats. In the liver of adult rats, there was also an increase in the p-Akt/Akt ratio ($P<0.05$), but in the kidney the ratio was not modified (Fig. 4A).

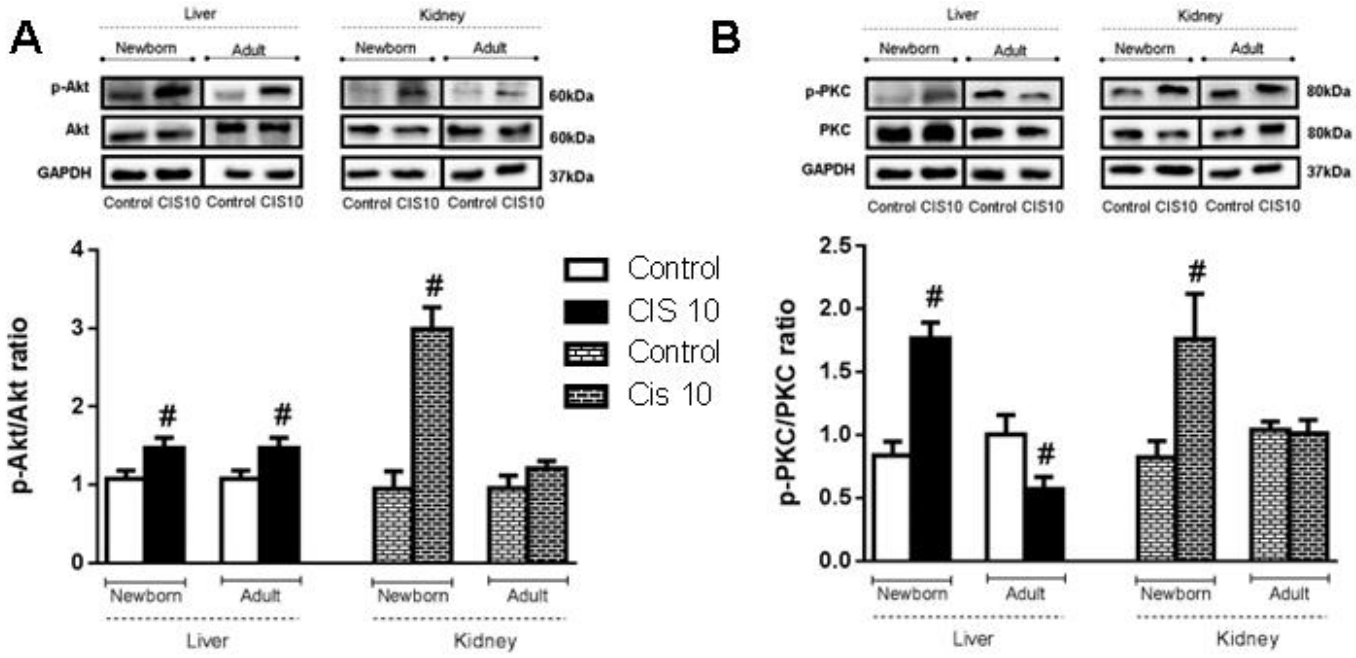

Figure 4. Effects of CIS administration on p-Akt/Akt ratio (A), p-PKC/PKC (B) in the liver and kidney of newborn and adult rats. Values are expressed as the mean ± SEM (n = 5 rats/group). Data were analyzed by unpaired Student's t test. # denotes the significance levels when compared to the control group. Photographs are representation of qualitative Western blotting analysis.

3.11 CIS affects phosphorylation of PKC

Unpaired Student's t test revealed that CIS-treated rats showed an increase of p-PKC/PKC ratio in the liver ($P<0.001$) and kidney ($P<0.05$) in newborn rats. However, in adult

rats the CIS administration decreased the p-PKC/PKC ratio in the liver (P<0.05) but not in the kidney (Fig.4B).

3.10 Comparison between newborn and adult control groups

Table 3 shows statistical analyses for comparison of data in newborn and adult control groups.

Table 3: Data on Newman-Keuls multiple comparisons test between newborn and adult control groups.

	Liver			Kidney		
	Mean Difference	F[5,30]	P	Mean Difference	F[5,30]	*P* value
GPx	433.2	15.01	<0.0001	303.2	52.65	<0.0001
GR	197.5	13.14	<0.01	-58.25	4.47	<0.05
GST	-2609	59,31	<0.0001	198.5	30.88	<0.0001
CAT	-9.915	26.56	<0.0001	-7.826	125.5	<0.0001
PC	7.322	40.42	<0.0001	9.116	23.34	<0.001
NPSH	1.123	37.37	<0.0001	1.256	76.97	<0.0001
δ-ALA-D	1.765	8.69	<0.01	-0.7204	13.68	<0.01

Data were analyzed using a One-way analysis of variance (ANOVA) followed by the Newman - Keuls test.

4 Discussion

The present results indicate that the highest dose of CIS led to hepatorenal toxicity in newborn and adult rats. However, the pattern of damage was different between ages and tissues. Newborn rats showed similar alterations in the liver and kidney, whereas the damage in adult rats was different in these tissues (Fig. 5). Moreover, newborn rats had differences in the basal levels in some of biomarkers analyzed when compared with those of adult rats (Table 3).

In the present study acute administration of CIS in newborn and adult rats caused toxic effects in the kidney and liver. Our findings are in agreement with those published in the literature in terms of acute injury because AST activity usually rise before ALT due to AST enzyme's peculiar intralobular distribution, especially the higher concentration of AST in zone 3 of the hepatic acinus [33]. Newborn rats showed an increase of 187% in the AST activity whereas 29% was increased in adult rats. Similar fact was observed in urea levels, a waste product and an important marker of acute kidney injury still used for monitoring the renal function in patients receiving treatment with CIS [34]. The increase in urea levels in newborn rats was almost twice that recorded in adult rats (109% x 58%).

It is well known that most of the toxic effects caused by CIS are related to its ability to increase RS generation and depletion of antioxidant systems, thus establishing an oxidative stress condition, disrupting the equilibrium among the production and elimination of oxidants [35]. In this study, an increase in RS levels, a reduction in CAT activity and the inhibition of δ-ALA-D, markers of oxidative stress and response to cell damage, were the only effects similar in liver and kidney of rats at both ages. All other parameters of oxidative stress evaluated in liver and kidney were differently affected by CIS in newborn and adult rats.

Regarding markers of oxidative damage, RS levels increased in liver and kidney of rats at both ages; although, the percentage of increase in newborn rats was of 22 and 44 higher than in liver and kidney of adults. The protein carbonyl content was increased only in newborn rats after CIS injection. Remarkably, our findings demonstrated that the newborn control rats also showed higher levels of protein carbonyl than adult control rats. Protein carbonyl groups are an accepted biomarker of oxidative stress due to their relative early formation and stability, also proteins are the most immediate target for suffering oxidative damage in the cells because their abundance and high rate constants for reaction [36]. CIS may have reduced the activity of antioxidant enzymes, such as CAT and SOD, through diminution of gene expression, or CIS can covalently bind to proteins, thus compromising their structures and consequently their functions are also affected [37]. CIS exposure reduced CAT activity in liver and kidney of both ages, but newborn rats exhibited higher reduction percentages in liver 45% versus 42% of adults and in kidney this difference was more pronounced 51% in newborn and 20% in adults. In addition, we also found differences between the basal levels because CAT activity in newborn rats was lower than in adult rats in both tissues. Another important mechanism of CIS-induced injury is the depletion of GSH levels, CIS forms complexes with thiol-containing molecules, such as GSH, which has the

crucial role of keeping SH-groups in the reduced state and providing the proton for the antioxidant enzymes [38]. As expected, the NPSH levels, an indirect measure of GSH levels, were reduced after CIS treatment in liver (31%) and kidney (32%) of newborn rats. However, our findings revealed two unexpected results: firstly, the NPSH levels were not changed in adult rats in both tissues; secondly, newborn control rats had higher levels of NPSH than adult control rats. The results on NPSH levels reiterate the importance of this defense mechanism in developmental organisms how is in newborn rats.

Sulfhydryl enzymes are also impaired by the attraction of CIS by sulfhydryl groups compromising its functionality, used as a marker of response to cell damage [39]. The reaction catalyzed by δ-ALA-D (porphobilinogen formation) is part of the route of synthesis of tetrapyrrolic compounds. Consequently, the reduction in the activity of this sulfhydryl enzyme implies in a pathological state resulting in the reduction/inhibition of the heme biosynthesis pathway, and as a consequence leaves to the accumulation of the ALA substrate, which could enhance RS production and exacerbate the development of oxidative stress [40]. In the present study, δ-ALA-D activity was inhibited by CIS in liver and kidney of adults and newborn rats. However, similar to that was observed in the CAT activity, newborn rats exhibited higher δ-ALA-D inhibition in liver 27% versus 20% than adults, and in kidney 39% in newborn and 23% in adults. Furthermore, when compared the difference between the control groups of both ages, newborn rats had a highest activity of δ-ALA-D in liver, but a lower activity in kidney. These findings also indicate discrepancy between ages, possibly because internal organs of newborn rats are still developing, rat kidneys for example reach maturity 15 days after birth [41]. Na^+, K^+-ATPase, a sulfhydryl enzyme, is crucial for metabolism of all animal cells, mainly for maintenance of electrolytic balance. The reduction of Na^+,K^+-ATPase activity in kidney can cause a hemodynamic dysfunction [5]. In this study, adult and newborn rats showed an inhibition of renal Na^+, K^+-ATPase activity, which could contribute to the development of renal injury.

Moreover, we previously demonstrated that CIS at a dose of 10 mg/kg causes impairment in enzymes of GSH redox cycle (GPx, GST and GR) of mice after three days of exposure [12]. Accordingly, the inhibition of GST activity in the liver of adult rats exposed to CIS was found in the present study. However, our results also reveal an increase in the GR activity in liver (12%) and kidney (77%) of newborn rats after CIS exposure. These results clearly point out physiological differences due to age on the effects of CIS in rats; because CIS decreased the GR activity in kidney (17 %) but did not alter the enzyme activity in the

liver of adult rats. Furthermore, the current data reveal that activities of GR and GPx are highest in newborn rats, these data are in agreement with those obtained by Frosali et al [42] that reported a greater GR activity in neonate than in adult humans. This leads us to believe that an increase in the GR activity is a more pronounced defense mechanism in newborn mammals.

Moreover, it is possible that in adult rats, different from newborns, GPx had a similar role to that of GR, because we found an increase in the GPx activity in liver and kidney of these animals, which was not found in newborn rats. These results reinforce our hypothesis that there is difference between newborn and adult rats in response to acute CIS exposure.

CIS interaction with DNA and generation of RS trigger apoptosis, which is marked by an increase of Bax levels, a pro-apoptotic protein, and a decrease of Bcl2, an anti-apoptotic protein [43]. This could induce a release of cytochrome c to cytosol and consequent activation of caspase pathway [38]. In the present study, CIS increased the levels of caspase-3 and the Bax/Bcl2 ratio in liver and kidney of newborn rats and in the liver of adult animals.

Nrf2, a transcription factor, is a major regulator of the adaptive response to oxidative stress in mammalian cells. Under physiological conditions, Nrf2 remains in cytoplasm, binds to Keap1 and quickly suffers ubiquitination and proteasomal degradation. Meanwhile in conditions of oxidative stress, however, the interaction between Nrf2 and Keap1 is disrupted, resulting in the accumulation of the former in the nucleus, where it interacts with antioxidant response elements (AREs) and promotes the expression of target genes [44]. Our current results demonstrate, at least at the first 24 h after CIS administration, a decrease in the Nrf2/Keap1 ratio in liver and kidney of newborn rats but alteration was not detected in both tissues of adult rats. Similar results were found recently by Forootan et al [45] who detected a Nrf2 stress response from 48 h after CIS administration at a dose of 20 mg/Kg in mice.

Because Nrf2 can be regulated by phosphorylation [44], we decided to investigate the levels of PKC and Akt proteins after exposure to CIS in rats. In addition to be aware that CIS has contrasting effects, either activation or down regulation of these proteins [38]. The present study found an increase in the ratios of p-Akt/Akt and p-PKC/PKC in liver and kidney of newborn rats. By contrast, a decrease in the p-PKC/PKC ratio and an increase in the p-Akt/Akt ratio in the liver, with no alteration in kidney, were found in adult animals. These findings led us to hypothesize that although the activation of Nrf2 had not yet taken place, previous steps (increase of the levels of protein kinases) had already begun after 24 h of CIS administration, mainly, in newborn rats.

In conclusion, the pattern of CIS toxic effects was different between ages and tissues. The newborn rats showed similar alterations in liver and kidney, such as oxidative damage and variations in protein levels involved with apoptosis and oxidative stress response. Furthermore, the current study revealed that newborn rats are more sensitive to CIS treatment, at least in the first 24 h; whereas in the beginning the damage was less severe in kidney than in liver of adult rats (Fig. 5). Thus, the present study reinforces the body response differences to CIS acute exposure between adult and newborn rats, strengthening the need for further studies about the children response to chemotherapy and possible effective strategies to minimize CIS damage.

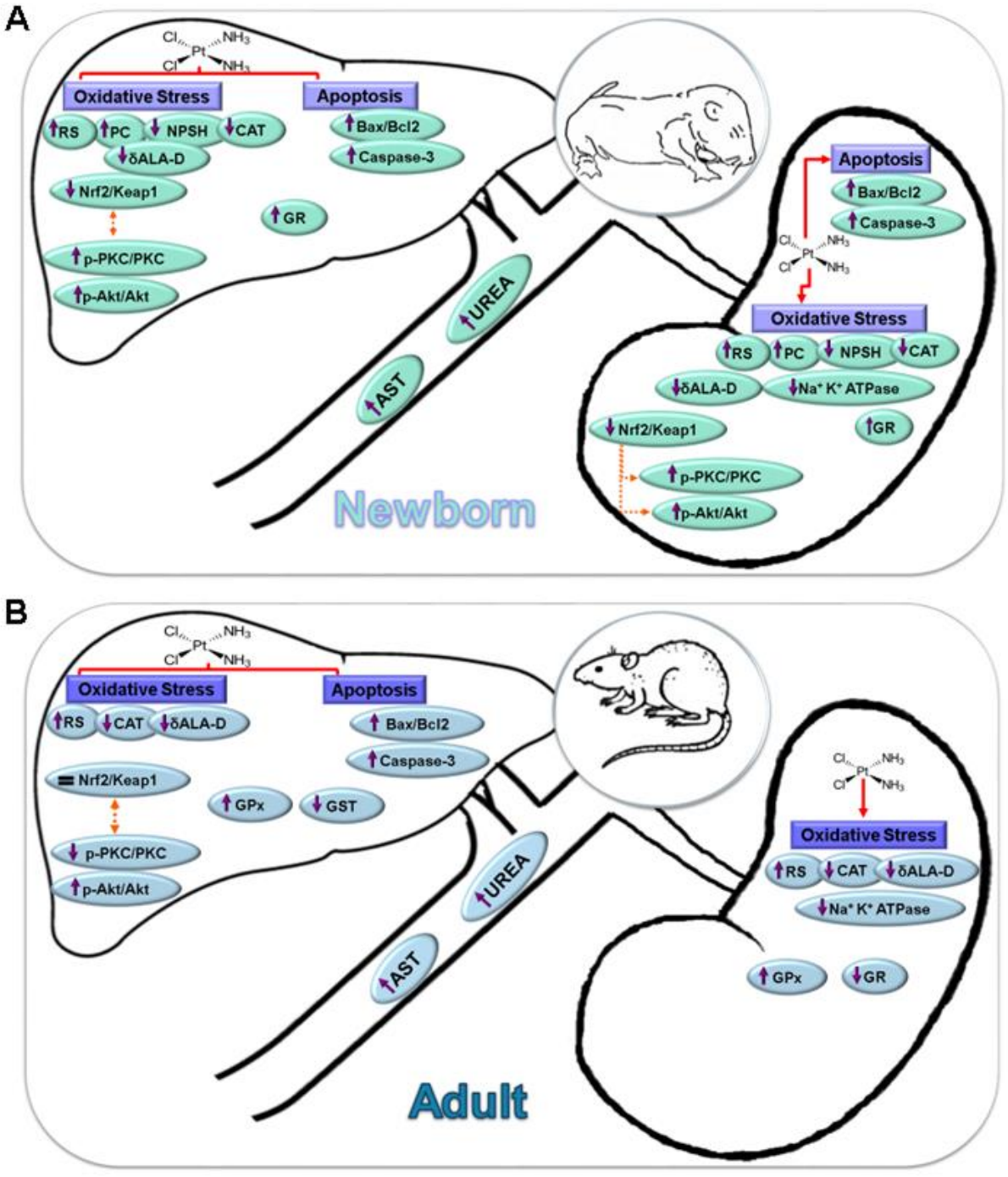

Figure 5. Summary of CIS main effects on newborn (A) and adult rats (B).

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Compliance with ethical standards: The experimental procedures were approved by the Institutional Animal Care and Use Committee of the Federal University of Santa Maria, Brazil and met the requirements of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Conflict of interest: The authors declare they have no conflicts of interest to disclose.

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4 CONCLUSÃO

Os dados deste estudo demonstram que a administração aguda de cisplatina causou toxicidade hepatorrenal em ratos recém-nascidos e adultos. No entanto, o padrão e a intensidade dos danos foram diferentes entre as idades e os tecidos avaliados. Ratos recém-nascidos apresentaram um dano no metabolismo oxidativo mais acentuado em relação aos ratos adultos, caracterizado por um aumento nas EROS e proteína carbonila, menor conteúdo de NPSH e maior inibição nas atividades das enzimas δ-ALA-D e CAT. Além disso, foi observada uma resposta molecular mais rápida nos níveis proteicos envolvidos com apoptose e resposta ao estresse oxidativo.

O presente estudo também revelou que os ratos recém-nascidos são mais sensíveis ao tratamento com a cisplatina, pelo menos nas primeiras 24 horas, e ainda mostrou que, pelo menos no início, o dano hepático foi maior do que o dano renal em ratos adultos. Assim, o presente estudo demonstrou que existem diferenças entre ratos adultos e recém-nascidos na resposta à exposição aguda a cisplatina, reforçando a necessidade de mais estudos sobre a resposta da criança à quimioterapia, e possíveis estratégias efetivas para minimizar os danos causados pela cisplatina.

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ANEXO A - CARTA DE APROVAÇÃO DO PROJETO DE PESQUISA PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE SANTA MARIA

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que o Projeto intitulado "Avaliação do Efeito Protetor de um Composto Orgânico de Selênio nos Danos Causados pela Cisplatina em Filhotes de Ratos", protocolado sob o CEUA nº 2699300315, sob a responsabilidade de **Cristina W. Nogueira** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei 11.794, de 8 de outubro de 2008, com o Decreto 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais Universidade Federal de Santa Maria (CEUA/UFSM) em reunião de 13/08/2015.

We certify that the proposal "Evaluation of the shield effect of a organoselenium compound on Damage Caused by cisplatin in rats puppies", utilizing 64 Heterogenics rats (64 males), protocol number CEUA 2699300315, under the responsibility of **Cristina W. Nogueira** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes (or teaching) - it's in accordance with Law 11.794, of October 8 2008, Decree 6899, of July 15, 2009, with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 08/13/2015.

Vigência da Proposta: de 05/2015 a 08/2016 Laboratório: Bioquímica E Biologia Molecular

Procedência:	Biotério Central UFSM				
Espécie:	Ratos heterogênicos	Gênero: Machos	idade:	10 dias	N: 64
Linhagem:	Wistar		Peso:	15g	

Nota: Um dos principais inconvenientes no tratamento do câncer são os danos toxicológicos causados pelos fármacos utilizados. Dentre essas drogas se destaca a Cisplatina (CIS), que é amplamente empregada na clínica, porém apresenta diversos efeitos colaterais. Por isso, o objetivo desse projeto é estudar os danos causados pela administração de cisplatina em filhotes de ratos Wistar, tentando assim simular o efeito deste medicamento quando utilizado em crianças. E ainda o efeito de um composto orgânico de selênio, a ser definido, quanto à proteção destes danos. Com esse propósito os animais serão divididos da seguinte maneira: Grupo I (salina); Grupo II (CIS 5 mg/kg i.p.); grupo III (10 mg/kg i.p.); Grupo IV (Composto de selênio + CIS 10 mg/kg). Investigaremos os parâmetros de estresse oxidativo e a atividade de enzimas sulfidrilicas para dimensionar esses danos em soro, fígado, rim e cérebro de ratos de 10 dias de idade.

Santa Maria, 10 de setembro de 2015

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

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CEUA Nº 2699300315