UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

NEUROPATIA DIABÉTICA: ESTUDO DOS MECANISMOS MOLECULARES ENVOLVIDOS COM A NEUROTOXICIDADE DO METILGLIOXAL E DO GLICOLALDEÍDO EM CÉLULAS DIFERENCIADAS DE NEUROBLASTOMA HUMANO SH-SY5Y

GIOVANA FERREIRA LONDERO

PORTO ALEGRE, MAIO DE 2012.

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PARTE I

RESUMO

Neuropatia é a complicação mais comum e mais debilitante da Diabetes Mellitus, a longo prazo presente em mais de 50% dos pacientes que possuem a doença. A hiperglicemia induz estresse oxidativo nos neurônios de diabéticos acarretando a ativação de múltiplas vias bioquímicas, as quais são potenciais alvos terapêuticos para a neuropatia diabética. Está claro que compostos carbonil reativos são mediadores glicotóxicos do estresse oxidativo através da formação de produtos finais de glicação avançada como resultado direto da hiperglicemia. Metilglioxal e glicolaldeído são compostos carbonil reativos inevitavelmente produzidos pelo metabolismo, os quais são encontrados em maior quantidade em situações de hiperglicemia. Recentemente, tem sido dada muita atenção para o envolvimento de espécies reativas na toxicidade do metilglioxal e do glicolaldeído, e tem-se demonstrado que essas glicotoxinas têm potencial para induzir estresse oxidativo, parar o crescimento celular e promover morte por apoptose ou necrose. O metilglioxal e o glicolaldeído interagem com grupamentos sulfidril de moléculas de glutationa e de enzimas, inibindo sua atividade; entretanto, os mecanismos moleculares relacionados aos efeitos tóxicos dessas glicotoxinas e as vias pelas quais elas levam a formação de espécies reativas não estão completamente elucidados. Neste estudo nós buscamos esclarecer a relação entre o metabolismo do metilglioxal e do glicolaldeído e a produção de espécies reativas, e investigamos as possíveis rotas de morte celular envolvidas. Utilizamos a linhagem celular de neuroblastoma humano SH-SY5Y diferenciada, pois este é um modelo neuronal bem caracterizado para estudos de compostos neurotóxicos. Nós avaliamos a produção de espécies reativas induzida por metilglioxal e glicolaldeído através da técnica da diclorofluoresceína, e avaliamos, também, seus efeitos sob o conteúdo de glutationa celular. Além disso, investigamos a ativação das caspase-3, -8 e -9 e a contribuição de diferentes sistemas peroxidases (glutationa-redutase e a tioredoxina-redutase), na defesa neuronal contra essas glicotoxinas. Como resultados encontramos que o tratamento com ambas glicotoxinas rapidamente provocou um aumento na produção de espécies reativas e diminuição do conteúdo de glutationa, com concomitante ativação das caspases-8 e -9 e, posteriormente, também houve ativação da caspase-3 pelo tratamento com metilglioxal. Vimos que a tioredoxina-redutase possui um papel mais importante na defesa celular contra a toxicidade do metilglioxal do que contra o glicolaldeído, enquanto que a glutationa-redutase tem papel semelhante na defesa celular contra ambas glicotoxinas. Nossos resultados demonstraram que o estresse oxidativo é um importante mecanismo da toxicidade do metilglioxal e do glicolaldeído nas células diferenciadas SH-SY5Y e, que enzimas redutoras de grupamentos sulfidril contribuem de diferentes formas na defesa celular contra cada uma dessas glicotoxinas.

Palavras-chave: Neuropatia diabética – Estresse Oxidativo – Metilglioxal – Glicolaldeído – Neurotoxicidade – Células SH-SY5Y

ABSTRACT

Neuropathy is the most common and debilitating complication of Diabetes Mellitus present in more than 50% of the patients with long-standing disease. Hyperglycemia induces oxidative stress in neurons from diabetic patients and results in activation of multiple biochemical pathways. These activated pathways are a major source of damage and are potential therapeutic targets in diabetic neuropathy. A large body of evidence has implicated reactive carbonyl compounds as glycotoxic mediators of oxidative stress by forming advanced glycation endproducts as a direct result of hyperglycemia. Methylglyoxal and glycolaldehyde are reactive carbonil compounds inevitably produced by the metabolism, but they are found in increased rates under hyperglycemia condition. Recently, the attention has been focused on the involvement of reactive species in methylglyoxal and glycolaldehyde toxicities, resulting in oxidative stress and leading to cell growth arrest, apoptotic or necrosis death. These glycotoxins interact with sulfhydryl-groups of glutathione molecules enzymes, inhibiting their activity; however, the molecular mechanism underlying methylglyoxal and glycolaldehyde cytotoxic effects and reactive species generation are not fully understood. In this study we have pursued to establish the role of methylglyoxal and glycolaldehyde metabolisms and reactive species production, and have looked for the possible death routes involved with the toxic effects of these glycotoxins. Here we used the differentiated human neuroblastoma SH-SY5Y cells as neuronal experimental model to investigate the pathological effects of various neurotoxic compounds. We have evaluated the methylglyoxal and glycolaldehyde capacity to reactive species generation by dichlorofluorescein assay and their effects upon cellular glutathione content. Also, we have assessed the caspase-3, -8 and -9 activation and the contribution of different peroxidases systems (glutathione reductase and thioredoxin reductase) in the neuronal defense against methylglyoxal and glycolaldehyde cytotoxicities. We found that both glycotoxins promptly provoke reactive species generation and decrease the cell glutathione content, as well induce caspase-8 and -9 activation. Later caspase-3 activation was found in methylglyoxal treatment. We demonstrate that thioredoxin reductase has a most important role in cell defense against methylglyoxal toxicity than against glycolaldehyde, meanwhile there is no difference in the glutathione reductase role. Our results show that oxidative stress is an important mechanism in the methylglyoxal and glycolaldehyde toxicities and sulfhydryl reductases contributes differently in the cellular defense against these glycotoxins.

Key words: Diabetes Neuropathy - Oxidative Stress - Methylglyoxal - Glycolaldehyde - Neurotoxicity - SH-SY5Y cells

LISTA DE ABREVIATURAS

6-OHDA: 6-hidroxidopamina

AGE: produto final de glicação avançada

AR: ácido retinóico

COX-2: ciclo-oxigenase-2

DAT: transportador de dopamina

DCF: diclorofluoresceína

DCNT: doenças crônicas não transmissíveis

DL_{50}: dose letal 50

DM: diabetes mellitus

ER: espécies reativas

FID: Federação Internacional de Diabetes

GA: glicolaldeído

GO: glioxal

GOLD: dímero glioxal-lisina

GPx: glutationa-peroxidase

GR: glutationa-redutase

GSH: glutationa reduzida

GSSH: glutationa oxidada

IP: iodeto de propídeo

LDH: lactato-desidrogenase

LO: lipo-oxigenase

MAPK: proteína cinase ativada por mitógeno

MG: metilglioxal

MTT: brometo de metiltiazolildifenil-tetrazolium

Na/K-ATPase: sódio/potássio-ATPase

ND: neuropatia diabética

NeuN: proteína nuclear específica de neurônio

NF-kB: fator de transcrição nuclear kappa B

NSE: enolase-neurônio-específica

OMS: Organização Mundial da Saúde

PARP: poli(ADP-ribose)-polimerase

PKC: proteína-cinase-C

RAGE: receptor para AGE

RAR: receptores ao ácido retinóico

RXR: receptores retinóides X

SOD: superóxido desmutase

TH: tirosina-hidroxilase

TrxR: tioredoxina-redutase

1. INTRODUÇÃO

1.1 A DIABETES MELLITUS

Desde a década de 60, observam-se processos de transição demográfica, epidemiológica e nutricional nos países latino-americanos, que resultaram em alterações nos seus padrões de morbimortalidade. Nos últimos anos, ocorreu acentuada queda da mortalidade, diminuição da fecundidade e aumento da expectativa de vida, acelerando o envelhecimento da população (CARDOSO e NAVARRO, 2007; CARVALHO; RODRIGUES-WONG, 2008). No Brasil observa-se uma grande diminuição na incidência de doenças infecciosas e parasitárias, e um aumento das doenças crônicas não transmissíveis (DCNT) (LUNA, 2002). Um estudo realizado pelo Ministério da Saúde demonstra que as DCNTs são responsáveis pela maior fração de anos de vida perdidos por morte prematura (59,0%) e de anos de vida perdidos por incapacidade (74,7%) (SCHRAMM *et al*., 2004). Dentre as DCNTs destaca-se a diabetes mellitus (DM) devido as suas altas taxas de morbimortalidade e pelas repercussões sociais e econômicas que acarreta; caracterizando-se, assim, como um dos principais problemas de saúde pública da atualidade (TORQUATO *et al.,* 2003; SOLLA, 2004).

Atualmente, cerca de 346 milhões de pessoas no mundo têm DM; e, segundo a Organização Mundial da Saúde (OMS), em 2004, essa doença foi responsável por 3,4 milhões de óbitos (WHO, 2010). No Brasil, havia aproximadamente 6,5 milhões de diabéticos em 2007 (VIGITEL, 2008), dos quais 50% desconheciam seu diagnóstico (IDF, 2008). A estimativa é que até

2030 o número de portadores de DM no Brasil chegue a 11 milhões, e o número de óbitos ocasionados por essa doença no mundo dobre (WHO, 2010).

O grande aumento na incidência da DM é o resultado de múltiplos fatores, tais como as mudanças socioculturais induzidas pela industrialização, o consumo de dietas hipercalóricas, a diminuição de práticas de atividade física, o aumento da obesidade, as mudanças no estilo de vida tradicional, e pelo aumento da expectativa de vida da população (AMERICAN DIABETES ASSOCIATION, 2007; OLIVEIRA *et al*., 2009). A DM, também, pode ser vista como um indicador macroeconômico, uma vez que atinge pessoas em plena vida produtiva (7,6% da população brasileira entre 30 e 69 anos) e acaba por onerar a previdência social e o sistema público de saúde (TORQUATO *et al.,* 2003; SOCIEDADE BRASILEIRA DE DIABETES, 2007). A Federação Internacional de Diabetes (FID) estima que em 2010 os custos globais com a doença foram de 376 bilhões de dólares, sendo as suas complicações crônicas as principais responsáveis por estes gastos (BOULTON *et al*., 2005).

1.2 FISIOPATOLOGIA DA DIABETES MELLITUS

A DM é uma doença metabólica formada por um grupo heterogêneo de alterações caracterizadas por hiperglicemia crônica associada com a ausência ou ação inadequada da insulina (SOCIEDADE BRASILEIRA DE DIABETES, 2008). É assinalada por complicações, disfunções e insuficiência de vários órgãos, que afetam especialmente os sistemas oftálmicos, renal, neurológico e cardiovascular. Essa doença resulta da diminuição da secreção e/ou da ação da insulina, envolvendo processos patogênicos específicos, como, por exemplo, destruição das células beta do pâncreas (produtoras de insulina),

resistência à ação da insulina, distúrbios da secreção da insulina, entre outros (SOCIEDADE BRASILEIRA DE DIABETES, 2007). A DM é influenciada por múltiplos e complexos fatores genéticos e ambientais, que interagem potencializando sua expressão patológica. O caráter hereditário da DM está relacionado com um gene regulador da produção de anticorpos anti-célula beta, localizado no braço curto do cromossomo seis, devendo haver, provavelmente, fatores ambientais que estimulam a sua expressão gênica mais precoce ou tardia, o que justifica as diferentes faixas etárias de manifestação da sintomatologia (MEHERS & GILLESPIE, 2008).

A ausência da insulina ou ineficiência de seus receptores impossibilita a captação de glicose pelas células sensíveis à insulina, levando ao seu acúmulo no sangue, o que caracteriza hiperglicemia. Os níveis de normalidade estão estabelecidos entre 70 e 110 mg/dL para glicemia de jejum de oito horas, a partir de ± 160 mg/dL há a extrapolação do limiar renal da glicose e a sua liberação na urina, o que caracteriza glicosúria. A ausência de glicose intracelular induz o fígado à gliconeogênese (produção de glicose através de precursores não glicídicos). Há, também, a mobilização dos ácidos graxos do tecido adiposo para produzir energia através da ß-oxidação, que fornecerá a energia necessária ao metabolismo celular. O acetil-CoA produzido pela ß-oxidação hepática excede a capacidade do ciclo de Krebs e é utilizado para a síntese de corpos cetônicos, que são utilizados como fonte de acetil-CoA pelos tecidos extra-hepáticos, e são eliminados pela respiração (hálito cetônico) e pela urina (cetonúria). O caráter ácido dos corpos cetônicos pode alterar o equilíbrio ácido-básico, levando o paciente ao coma e morte (AMERICAN DIABETES ASSOCIATION, 2008; GALLAGHER *et al.,* 2011).

Baseada na etiologia, a DM pode ser denominada como: tipo 1, tipo 2, gestacional e outros tipos específicos (SOCIEDADE BRASILEIRA DE DIABETES, 2007). Por DM do tipo 1, entende-se a manifestação clínica da DM onde, por mecanismos variados, as células beta do pâncreas são danificadas e a produção de insulina diminui consideravelmente ou cessa. Como consequência, a glicose não penetra na célula, levando à hiperglicemia e todos os efeitos derivados a este fato, que compõem a fisiopatologia clássica da doença, obrigando o paciente a tornar-se insulinodependente (GALLAGHER *et al.,* 2011). A DM tipo 1 representa de 5% a 10% dos casos, podendo ser imunomediada ou idiopática e, aparece, na maioria das vezes, em pessoas jovens (menores de 35 anos), com deficiência total ou quase total da insulina produzida pelo pâncreas. É considerada uma doença auto-imune quando são identificados anticorpos específicos que levam a destruição das células beta pancreáticas. Quando não identificados tais mecanismos, é considerada idiopática (SOCIEDADE BRASILEIRA DE DIABETES, 2007).

O DM tipo 2 representa de 90% a 95% dos casos e ocorre, comumente, após os 40 anos de idade, mantendo uma evolução lenta. Esse tipo de DM acomete com maior frequência indivíduos com sobrepeso, obesos e com histórico familiar da doença (AMERICAN DIABETES ASSOCIATION, 2004; SOCIEDADE BRASILEIRA DE DIABETES, 2007). Em seu estágio inicial, a DM tipo 2 é assintomática, o que retarda seu diagnóstico por muitos anos, favorecendo, com isso, o desenvolvimento de complicações crônicas, que conduzem ao diagnóstico somente por suas manifestações clínicas, as vezes irreversíveis (SOCIEDADE BRASILEIRA DE DIABETES, 2007; AMERICAN DIABETES ASSOCIATION, 2008). O processo fisiopatológico do DM tipo 2

caracteriza-se pela resistência insulínica, em que o nível de insulina é normal, mas não é eficaz em sua função de promover a utilização periférica da glicose (SOCIEDADE BRASILEIRA DE DIABETES, 2007). O pâncreas segue produzindo insulina, porém essa não é suficientemente ativa em nível celular. Nesse tipo de DM, a principal disfunção reside nos receptores insulínicos celulares, que não têm o número ou a forma adequados para que a insulina possa agir sobre eles, permitindo a captação da glicose nas células insulino-dependentes (GALLAGHER *et al.,* 2011). Os pacientes apresentam excesso de glicose no sangue (hiperglicemia), mas os níveis de insulina podem estar normais ou mesmo aumentados, fazendo com que, de modo contrário ao que acontece no DM tipo 1, geralmente não se faça necessário o uso habitual de insulina exógena. Uma segunda condição é a insuficiência insulínica, situação na qual existe efetivamente uma redução de sua secreção pelo pâncreas e que, geralmente, ocorre depois de alguns anos de evolução da doença (PEDROSA *et al.,* 2006). As pessoas com essa forma do DM não são dependentes de insulina exógena para sobrevivência, porem podem necessitar de tratamento com insulina para a obtenção de um controle metabólico adequado (AMERICAN DIABETES ASSOCIATION, 2008; SOCIEDADE BRASILEIRA DE DIABETES, 2007; GALLAGHER *et al.,* 2011)).

A DM gestacional, que ocorre em 1% a 14% de todas as gestações, dependendo da população estudada, está associada ao aumento da morbidade e mortalidade perinatal (PEDROSA *et al.,* 2006). Na maioria dos casos, há reversão para a tolerância normal a glicose após a gravidez, porém existe um risco de 17% a 63% de desenvolvimento de DM tipo 2 pela mãe dentro de 5 a 16 anos apos o parto (HANNA & PETERS, 2002). Os outros tipos específicos

de DM são formas menos comuns, que podem ser causadas por defeitos genéticos, processos patológicos, infecções e exposição a drogas ou substâncias químicas (AMERICAN DIABETES ASSOCIATION, 2008; SOCIEDADE BRASILEIRA DE DIABETES, 2007).

Independentemente do tipo de DM, uma das maiores preocupações dos profissionais de saúde é a prevenção de complicações, devido a sua gravidade e repercussões para o paciente, família e sociedade. As complicações do DM são classificadas em agudas e crônicas. Entre as complicações agudas encontram-se a cetoacidose diabética e o coma hiperosmolar não cetótico, complicações essas de fácil manejo clínico, mas que podem ter consequências sérias se não tratadas a tempo (PEDROSA *et al.,*2006). As complicações crônicas do DM incluem as alterações vasculares e as neuropatias (GALLAGHER *et al.,* 2011).

1.3 A NEUROPATIA DIABÉTICA

A neuropatia diabética (ND) é a complicação mais frequente da DM e é o seu principal fator de morbidade e mortalidade (BASIĆ-KES *et al.,* 2011; UZAR *et al.,* 2011). A ND consiste em um grupo de síndromes clínicas e subclínicas de etiologia, manifestação clínica e laboratorial variadas; caracterizadas por dano difuso ou focal das fibras nervosas periféricas somáticas ou autonômicas, podendo acometer qualquer fibra nervosa do corpo humano (GOMEZ *et al.,* 2002). Estima-se que mais de 50% dos diabéticos desenvolverão ND após 25 anos de doença (GALLAGHER *et al.,* 2011) e, ainda que essa complicação seja apenas subclínica, em quase 100% dos casos as alterações eletrofisiológicas indicativas de ND estão presentes (ROSENSTOCK *et al.,*

2004). Além disso, quando há ND, 10% das pessoas com DM tipo 1 e 20% das com tipo 2 apresentam dor grave (GALER *et al.,* 2000; ROSENSTOCK *et al.,* 2004). A dor neuropática diabética tem intensidade moderada-grave (GALER *et al.,* 2000; DAVIES *et al.,* 2006) e é descrita como formigamento, queimação, contínua, lacerante, com sensação de agulhadas, localizada distal, bilateral e simetricamente, com alterações sensoriais anormais, como a alodínia ou hiperalgesia (GALER *et al.,* 2000; SOCIEDADE BRASILEIRA DE DIABETES, 2007; TALIYAN & SHARMA, 2012). O tratamento da dor é complexo, utilizando fármacos antineuríticos como os antidepressivos tricíclicos, anticonvulsivantes, simpatomiméticos e agentes tópicos. A melhora da funcionalidade nervosa e o bloqueio da transmissão dos impulsos dolorosos ainda são pouco satisfatórios e os efeitos adversos destes fármacos são inúmeros (JUDE & SCHAPER, 2007; WONG *et al.,* 2007).

As desordens neuropáticas incluem manifestações somáticas e/ou do sistema nervoso autonômico. Sua natureza é heterogênea e vários mecanismos estão envolvidos na sua patogênese; entretanto, a hiperglicemia crônica e as desordens metabólicas a ela associadas são de alguma forma implicadas em quase todos os tipos de ND (GALLAGHER *et al.,* 2011). A ND pode ocorrer de forma subclínica, que consiste na evidência de disfunção de nervos, tal como condução nervosa sensorial ou motora diminuída ou limiar sensorial elevado, que ocorre na ausência de sinais clínicos e sintomas de ND; ou, na forma clínica, que consiste na superposição de sintomas e/ou deficiências neurológicas clinicamente detectáveis (STAVNIICHUK *et al.,* 2012).

1.4 MECANISMOS PATOLÓGICOS DAS NEUROPATIAS

Apesar dos recentes avanços na compreensão dos mecanismos patológicos para muitas das complicações que ocorrem a longo prazo na DM, a patogênese das NDs não está completamente esclarecida (UZAR *et al.,* 2011; ZHANG *et al.,* 2012). Os mecanismos prováveis de lesão à fibra neurológica são: lesão metabólica direta, lesão metabólica provocando insuficiência neurovascular, perda de fatores neurotróficos e lesão auto-imune (EDWARDS *et al.,* 2008; RUSSELL *et al.,* 2008). Interações entre várias consequências metabólicas diretas e indiretas da deficiência de insulina, ou hiperglicemia, associadas a fatores ambientais e genéticos não completamente definidos, são necessários para o surgimento das NDs da DM.

A patogênese da ND tem sido extensivamente estudada em modelos animais de DM e, apesar de nenhum dos modelos existentes reproduzir fielmente as modificações encontradas na ND em humanos, os resultados mostram que existem interações complexas entre mecanismos vasculares e não-vasculares (CAMERON *et al.,* 2001; OBROSOVA *et al.,* 2009). Foram descritas múltiplas alterações bioquímicas, como atividade aumentada da via do sorbitol no metabolismo da glicose (YAGIHASHI *et al.,* 2001; OBROSOVA *et al.,* 2002), glicação e glico-oxidação não-enzimática de biomoléculas (CAMERON *et al.,* 2005; TOTH *et al.,* 2008;), ativação da proteína cinase C (PKC) e proteína cinase ativada por mitógeno (MAPK) (PRICE *et al.,* 2004; CHENG *et al.,* 2010), estresse oxidativo e nitrosativo (CAMERON *et al.,* 2001; COPPEY *et al.,* 2001; OBROSOVA *et al.,* 2001), déficits de fatores neurotróficos (LEHMANN *et al.,* 2010), ativação da poli(ADP-ribose)-polimerase (PARP) (LI *et al.,* 2005; OBROSOVA *et al.,* 2008), assim como, de enzimas do

metabolismo do ácido araquidônico, ciclo-oxigenase-2 (COX-2) (KELLOGG *et al.,* 2007) e lipo-oxigenase (LO) (STAVNIICHUK *et al.,* 2010; OBROSOVA *et al.,* 2010), participam no desenvolvimento dos déficits de condução nervosa e disfunção das fibras sensoriais.

Aumento na atividade da via do sorbitol (KATO *et al.,* 2000; HO *et al.,* 2006), déficit de fatores neurotróficos (TOTH *et al.,* 2006; FRANCIS *et al.,* 2009), estresse oxidativo e nitrosativo (HOUNSOM *et al.,* 2001), ativação da PARP (DREL *et al.,* 2010), COX-2 (KELLOGG *et al.,* 2007), e LO (OBROSOVA *et al.,* 2010) também têm sido relacionados com a atrofia axonal das fibras grandes mielinizadas e com a degeneração das pequenas fibras sensoriais. Muitos mecanismos bioquímicos têm sido estudados, assim como, a interação entre eles (OBROSOVA *et al.,* 2002; OBROSOVA *et al.,* 2005; HO *et al.,* 2006; DREL *et al.,* 2006; KUZUMOTO *et al.,* 2006; ASKWITH *et al.,* 2009); porém, muitos outros permanecem inexplorados (STAVNIICHUK *et al.,* 2012).

A hipótese metabólica conhecida como via dos polióis, busca explicar a ND a partir dos distúrbios metabólicos encontrados. Nos neurônios, a glicose é convertida em sorbitol e frutose pelas enzimas aldose-redutase e sorbitol-desidrogenase (STAVNIICHUK *et al.,* 2010). A hiperglicemia levaria a um acúmulo de sorbitol e frutose nos nervos periféricos, assim como em outros tecidos. O acúmulo de sorbitol conduz a uma cascata de eventos onde se observa redução no nível de mioinositol (talvez por uma compensação ao aumento de osmolaridade provocada pelo acúmulo de sorbitol), do *turnover* dos fosfolipídeos, da atividade da Na/K-ATPase da membrana com consequente acúmulo de sódio intra-axonal, reduzindo também a velocidade de condução nervosa e, por fim provocando rompimento da estrutura do nervo

(CAMERON *et al.,* 1999; STAVNIICHUK *et al.,* 2010;). Além disso, o acúmulo de frutose leva a uma redução na relação NADH/NAD, levando a célula a uma situação denominada de pseudo-hipóxia (OBROSOVA *et al.,* 2005); e, sendo a frutose um substrato dez vezes mais potente para a glicosilação do que a glicose, provoca um acúmulo de produtos finais de glicação avançada (AGEs). Outra consequência dessa cascata de eventos é a redução dos níveis celulares de NADPH, diminuindo a reciclagem da glutationa (GSH), o que gera uma menor captação de espécies reativas (ER) (BROWNLEE *et al.,* 2005).

Têm sido sugeridas várias fontes geradoras de ER na DM, como a NADPH-oxidase endotelial, a xantina-oxidase, óxido-nítrico-sintase e a ineficiência da cadeia respiratória mitocondrial (CAMERON & COTTER, 1999). Além disso, a DM está associada com a redução da atividade da Cu/Zn-superóxido-dismutase (CUI *et al.,* 2008), glutationa-peroxidase (GPx) (YU *et al.,* 2006), diminuições nos níveis de GSH (ARORA *et al.,* 2008), vitamina E (MANZELLA *et al.,* 2001), L-carnitina (IDO *et al.,* 1994) e aumentos nos produtos de peroxidação lipídica como o malondialdeído ou dienos conjugados (CUNHA *et al.,* 2008).

Evidências, de múltiplas fontes, indicam que o estresse oxidativo consequente da geração de ER pela hiperglicemia é um importante mecanismo envolvido tanto no desenvolvimento, como na progressão da ND (STEVENS *et al.,* 2000; OBROSOVA, 2002; RUSSELL *et al.,* 2002; SCHMEICHEL *et al.,* 2003; VINCENT & FELDMAN, 2004; RUSSELL *et al.,* 2008; KAMBOJ *et al.,* 2010). Existem estudos indicando a ocorrência de estresse oxidativo sistêmico em ratos diabéticos (CHINEN *et al.,* 2007; OLTMAN *et al.,* 2005; SERKOVA *et al.,* 2006; SONTA *et al.,* 2004); entretanto, a relação entre o estresse oxidativo

e os mecanismos de desenvolvimento da ND ainda não estão bem estabelecidos.

Os mecanismos envolvidos no aumento do estresse oxidativo na DM incluem, não apenas a geração de ER pela glicação não-enzimática e auto-oxidação de produtos de glicação, mas também, mudanças no conteúdo e na atividade dos sistemas de defesa antioxidantes (EDWARDS *et al.,* 2008). Aumentos nos níveis de produtos lipídicos oxidados têm sido detectados no soro de pacientes diabéticos e estão possivelmente relacionados com as complicações dessa doença (EL BOGHDADY *et al.,* 2012). Já foi relatado que mudanças nos níveis de citocinas pró-inflamatórias, ER e AGEs gerados pela hiperglicemia podem contribuir para a ativação do fator de transcrição nuclear kappa B (NF-kB), que é redox-sensível, e este por sua vez, ativa genes de citocinas, moléculas de adesão, endotelina-1 e fator tecidual, o que dificulta a defesa celular contra o estresse oxidativo (KUMAR *et al.,* 2012). Outro achado interessante é que a diminuição da velocidade de condução nervosa, característica clínica da ND, está associada na maioria das vezes a uma diminuição da atividade da Na/K-ATPase dos neurônios, como consequência do estresse oxidativo (CAMERON *et al.,* 1999; KAMBOJ *et al.,* 2009).

Certamente a disfunção mitocondrial induzida pelo estresse oxidativo gerado pela hiperglicemia contribui para a sensibilização do neurônio (ZHANG *et al.,* 2012). Sob condições hiperglicêmicas, o fluxo metabólico mitocondrial aumentado aliado a desregulação da bomba de prótons resulta em um aumento na formação de ER, incluindo radicais peroxinitrito, superóxido e hidroxil (BROWNLEE, 2001; VINCENT *et al.,* 2002; BROWNLEE, 2005). Essas ER estão relacionadas com a peroxidação dos lipídeos de membrana, nitração

de proteínas e danos às moléculas de DNA (VINCENT & FELDMAN, 2004). Danos oxidativos mitocondriais mais a diminuição da ação da insulina e do suporte neurotrófico contribuem para a despolarização da membrana mitocondrial, *swelling* da mitocôndria, liberação do citocromo *c* no citosol e ativação de caspases em neurônios ganglionares dorsais (RUSSELL *et al.,* 2002; SCHMEICHEL *et al.,* 2003; VINCENT *et al.,* 2004); estando, já, todas essas características associadas a danos neuronais e axonais (KISHI *et al.,* 2002; HUANG *et al.,* 2003; RUSSEL *et al.,* 2008). Estudos *in vitro* mostraram que tanto a inibição da geração de ER induzida pela glicose a nível de cadeia transportadora de elétrons, como a estabilização do potencial de membrana mitocondrial, bloqueiam os danos aos neurônios sensoriais (RUSSELL *et al.,* 2002; LEINNINGER *et al.,* 2004; VINCENT & FELDMAN, 2004; RUSSELL *et al.,* 2012). Tratamentos com insulina ou antioxidantes como ácido α-lipóico (JIN *et al.,* 2007), GSH (UENO *et al.,* 2002), eritropoietina (BIANCHI *et al.,* 2004), quelantes de metais de transição como desferroxamina e trientina (CAMERON & COTTER, 1995), vitamina E (SKALSKA *et al.,* 2008), ácido docosaexaenóico (COSTE *et al.,* 2003), N-acetilcisteína (KAMBOJ *et al.,* 2010) estão associados com melhorias na função das células nervosas; porém, outros antioxidantes clássicos falharam em mostrar benefícios efetivos (SHELTON *et al.,* 2005).

1.5 AS GLICOTOXINAS GLIOXAL, METILGLIOXAL E GLICOLALDEÍDO

Os processos envolvidos no dano a células neuronais pela hiperglicemia são complexos e provavelmente envolvem várias características como excitotoxicidade, disfunção mitocondrial, agregação proteica anormal, e inflamação; entretanto a formação de ER é um processo relacionado a todos

esses fenômenos (EDWARDS *et al.,* 2008). Formados endogenamente como produto intermediário da via glicolítica ou, também, de forma não-enzimática pelas reações de fragmentação de açúcares (THORNALLEY, 1996), compostos carbonil reativos, como o metilglioxal (MG), glioxal (GO) e glicolaldeído (GA) devem contribuir para a toxicidade da hiperglicemia não controlada (SHUVAEV *et al*., 1998; GARCÍA DE ARRIBA *et al*, 2006), como ilustrado na figura 1.

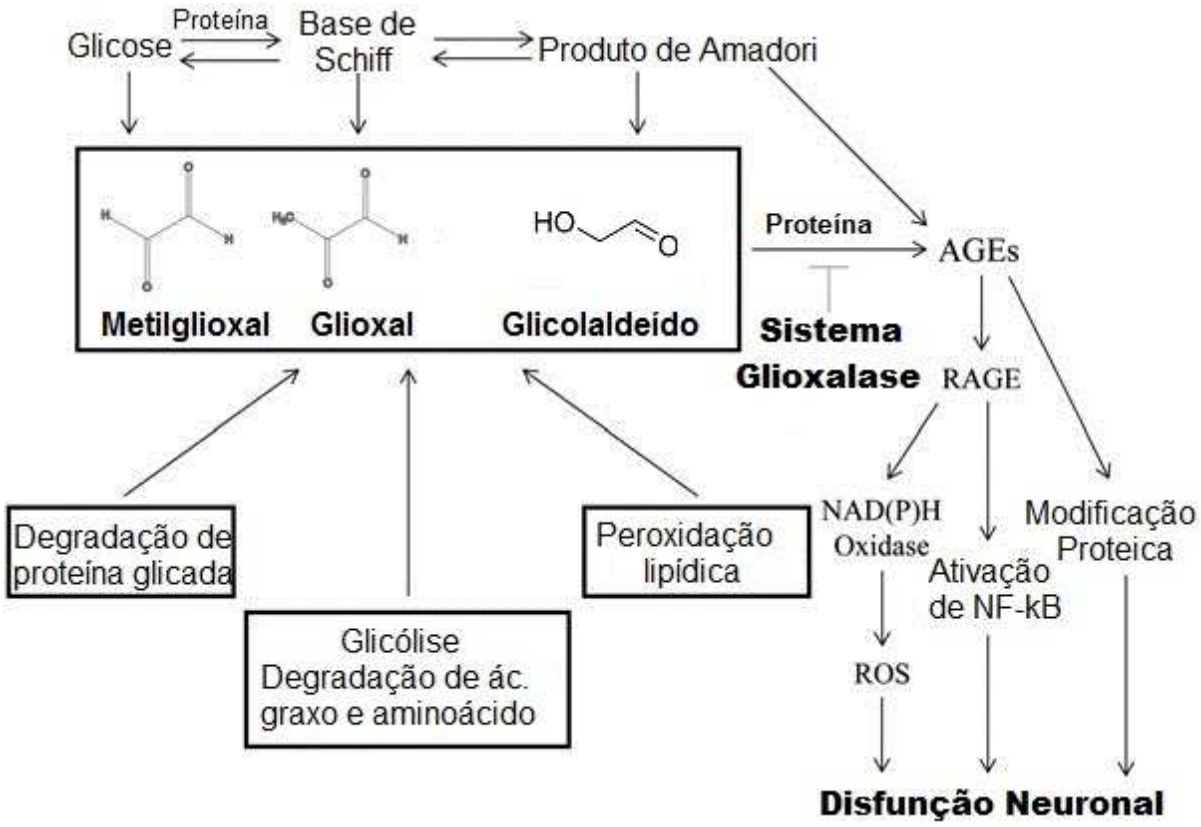

Fig. 1: O esquema ilustra as vias que levam a produção de compostos carbonil reativos e AGEs, e os mecanismos pelos quais os AGEs causam disfunção neuronal. (Adaptado de JACK & WRIGHT, 2011) AGE: produtos finais de glicação avançada, RAGE: receptor de AGE, NF-kB: fator de transcrição nuclear kappa B, ER: espécies reativas.

O α-cetoaldeído MG é um dos compostos carbonil mais estudados, ele acumula em condições como hiperglicemia ou quando existem impedimentos no metabolismo normal da glicose; causando estresse oxidativo (GARCÍA DE ARRIBA *et al.*, 2007). O MG é um intermediário reativo da rede metabólica, sendo sempre produzido pelo metabolismo, mesmo em condições normais. Existem muitas vias para sua produção, envolvendo reações catalisadas enzimática e não-enzimaticamente (PHILLIPS & THORNALLEY, 1993a; RICHARD, 1991). O MG é formado pela fragmentação e eliminação do fosfato da forma fosfo-eno-diolato do gliceraldeído-3-fosfato e da diidroxiacetona-fosfato. Isto pode acontecer enzimaticamente pelo vazamento do fosfo-eno-diolato do sítio ativo da triosefosfato isomerase (POMPLIANO *et al.*, 1990) e pela metilglioxal-sintase (RAY & RAY, 1981).

Também, pode ser formado a partir da acetona pelo citocromo P450 2E1, o qual catalisa a formação sequencial da hidroxiacetona e MG (REICHARD *et al.*, 1986; KOOP & CASAZZA, 1985), e pela oxidação da aminoacetona formada no catabolismo da treonina (LYLES & CHALMERS, 1992). Entretanto, a principal fonte de MG é a fragmentação não-enzimática de triosefosfatos (PHILLIPS & THORNALLEY, 1993b). A sua detoxificação se dá pelo sistema glioxalase através da catálise da GSH, compreendendo duas enzimas: a glioxalase I e II (MANNERVIK & RIDDERSTRÖM, 1993), como ilustrado na figura 2.

Sabe-se que o MG interage com os grupamentos sulfidril das proteínas e que forma adutos hemetioacetal com a GSH. Devido a essa alta reatividade com sulfidrilas ele acaba inibindo a atividade de muitas enzimas, inclusive das que participam da eliminação de radicais livres.

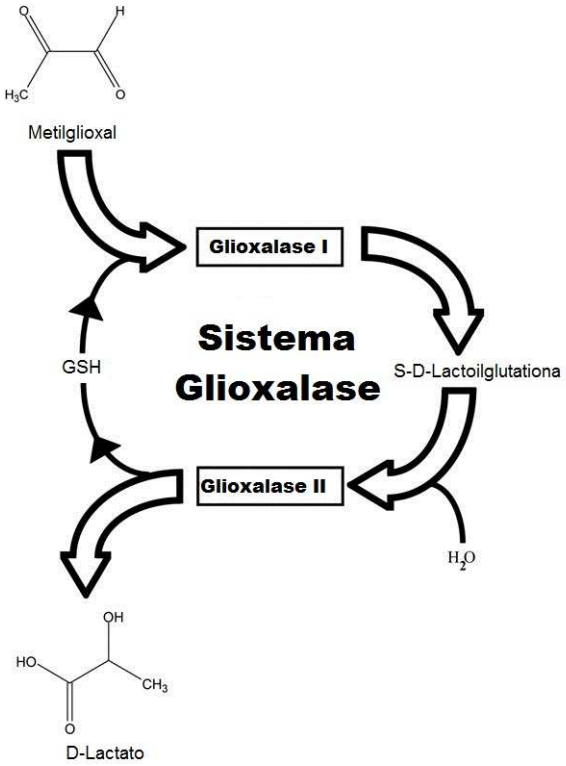

Fig. 2: O sistema glioxalase é composto por duas enzimas, glioxalase I e glioxalase II. Compostos diacarbonil reativos, como MG e GO, são efetivamente detoxificados através dessa via metabólica. A enzima glioxalase catalisa a conversão de α-oxoaldeídos no correspondente α-hidroxiácidos. Nesta figura esquemática, MG reage com GSH e é convertido a S-D-Lactoilglutationa pela glioxalase I. Depois, este intermediário é hidrolisado em D-lactato pela glioxalase II e a GSH é reciclada. (JACK & WRIGHT, 2011)

Assim, quando presente em altas concentrações, este composto carbonil reativo acaba depletando GSH, o qual tem um papel importantíssimo na proteção da célula contra danos oxidativos, e inibindo as enzimas antioxidantes, o que leva a célula a uma situação de estresse oxidativo (KALAPOS *et al*., 2008). Existem diversos estudos mostrando que pré-tratamentos com GSH, aminoguanidina ou N-acetilcisteína podem proteger as células do estresse oxidativo e dos danos celulares gerados pelo MG (WU & JUURLINK, 2002; AMICARELLI ET AL, 2003; FUKUNAGA *et al*., 2004).

A sua exata concentração intracelular nos neurônios ainda não é conhecida, mas o MG tem sido encontrado em fluidos corporais e tecido de pacientes e animais diabéticos em uma concentração de cerca de 400 μM (Lapolla et al., 2003a). Quanto aos neurônios, a exata concentração intracelular de MG ainda não é conhecida. Concentrações por volta de 300 μM foram mensuradas na linhagem de células CHO derivadas de ovário de hamster chinês, a maior parte ligado a resíduos de cisteína e GSH (GARCÍA DE ARRIBA *et al*., 2007).

O GA, um α-hidroxialdeído altamente reativo, é produzido pela fragmentação de açúcares nos estágios iniciais da glicosilação não-enzimática e é responsável por parte da produção de AGEs, podendo levar, muitas vezes, a complicações diabéticas (AL-ENEZI *et al*., 2006). Sua formação pode se dar pela degradação oxidativa da glicose a partir de proteínas glicadas, peroxidação lipídica, oxidação de aminoácidos e pelos neutrófilos durante a fagocitose (AL-ENEZI *et al*., 2006). Devido ao fato de que o grupo carbonil do GA não pode ser bloqueado por ciclização, ele é suscetível a enolização seguida de oxidação com concomitante produção de GO e radical superóxido (SAKURAI & TSUCHIYA, 1988).

O GO é altamente reativo e pode interagir com proteínas, lipídios e ácidos nucléicos (KASPER *et al*., 2000; THORNALLEY, 2002; SHANGARI *et al*., 2003; THORNALLEY, 2003). Ele também é formado como produto da peroxidação lipídica, sendo um importante agente glicador nos sistemas fisiológicos e formador de AGEs (TURK, 2010). Devido a sua alta reatividade com proteínas, a sua mensuração em amostras biológicas é bastante difícil de ser feita (NAKAJIMA *et al.,* 2007); entretanto, existem estudos que detectaram

este composto carbonil em altos níveis no plasma de pacientes diabéticos (LAPOLLA *et al.,* 2003b, HAN *et al.,* 2007). Existe um AGE que é especificamente derivado do GO, o dímero glioxal-lisina (GOLD); que é formado através do *crosslinking* de duas moléculas de lisina e uma de GO. O GOLD tem sido encontrado em grandes quantidades em pacientes diabéticos (SHAMSI & NAGARAJ 1999; SADY *et al.,* 2000).

Reações não enzimáticas entre açúcares redutores ou oxaldeídos e proteínas/lipídeos resultam em AGEs (AHMED, 2005; TOTH *et al.,* 2008). Três vias principais são responsáveis pela formação de radicais dicarbonis reativos (precursores de AGEs): a oxidação da glicose para formar GO; a degradação de produtos de Amadori (adutos frutose-lisina); e o metabolismo aberrante de intermediários glicolíticos do MG (EDWARDS *et al.,* 2008). AGEs são biomoléculas modificadas de forma heterogênea, intra ou extracelularmente. Dentro das células tanto adutos proteicos, como de DNA alteram a função e transporte celular. AGEs proteicos extracelulares podem danificar proteínas plasmáticas e de matriz, rompendo adesões celulares e ativando o receptor para AGEs (RAGE) (RAMASAMY *et al.,* 2007). A interação AGE-RAGE ativa a transcrição do NF-kB, o qual está relacionado ao processo inflamatório e a ativação da apoptose (RAMASAMY *et al.,* 2005). Também, a ativação de RAGE neuronal induz estresse oxidativo devido ao aumento da atividade da NADPH-oxidase (VINCENT *et al.,* 2007). Níveis aumentados de AGEs e RAGE foram encontrados em estudos feitos com tecido de humanos diabéticos (TANJI *et al.,* 2000). Camundongos diabéticos *knockout* para RAGE mostraram uma significante melhora na ND e diminuída expressão de NF-kB e PKC comparados com o modelo de animais *wildtype* (TOTH *et al.,* 2008). Com uma

visão geral, pode-se dizer que os danos bioquímicos induzidos por AGEs resultam em fluxo sanguíneo nervoso insuficiente, suporte neurotrófico diminuído e diminuição do transporte axonal (WADA & YAGIHASHI, 2005). A glicação é um processo endógeno que participa das modificações pós-traducionais das proteínas. O estágio inicial da glicação envolve a reação da glicose com o grupo amino de resíduos de lisina das proteínas para formar bases de Schiff e frutosaminas (THORNALLEY *et al*., 1999), que são posteriormente convertidos em AGEs. A glicação e a formação de AGEs têm sido muito estudadas em relação a DM e as complicações relacionadas a DM; mas, está claro que a glicação e os AGEs também têm relação as doenças neurodegenerativas como Alzheimer, Parkinson e esclerose amiotrófica lateral (KIKUCHI *et al*., 1999). A formação de AGEs tem sido caracterizada como um marcador intracelular da doença de Alzheimer nos emaranhados neurofibrilares (WONG *et al*., 2001) e doença de Parkinson nos corpos de Lewi (LO *et al*., 2004), sugerindo que estes depósitos proteicos foram expostos a precursores de AGEs. Existem estudos prévios que investigaram o mecanismo de indução de morte celular em células de neuroblastoma SH-SY5Y por AGEs (DEUTHER-CONRAD *et al*., 2001; GARCIA DE ARRIBA *et al*., 2003). Nestes estudos, a toxicidade dos AGEs foi prevenida por aminoguanidina, um *scavenger* de carbonil já bem estabelecido. Devido ao fato de compostos carbonil serem mediadores comuns de estresse oxidativo em eventos patológicos presentes em doenças neurodegenerativas, foi sugerido que o uso de antioxidantes combateria os efeitos negativos gerados por eles (DURANY *et al*., 1999; KIKUCHI *et al*., 1999).

Doenças neurodegenerativas como Alzheimer e Parkinson, e a DM estão associadas com uma formação aumentada de AGEs (GARCÍA DE ARRIBA *et al*., 2006) e, portanto, com o acúmulo de MG, o qual é o mais importante precursor fisiológico de AGEs (AHMED *et al*., 2003; AHMED *et al*., 2005). Entretanto, a contribuição da toxicidade do MG e outras glicotoxinas para a degeneração neuronal ainda não está bem compreendida.

1.6 MODELO EXPERIMENTAL: A LINHAGEM SH-SY5Y

A linhagem de neuroblastoma humano SH-SY5Y é um subclone derivado do neuroblastoma SK-N-SH. Essas células, quando em cultura, se assemelham com neuroblastos do sistema simpático, apresentando morfologia epitelial com citoplasma escasso (BIEDLER *et al.,* 1978). Células dessa linhagem estão estacionadas nos estágios iniciais da diferenciação neuronal e são caracterizadas bioquimicamente pela escassez de marcadores neuronais (CONROY & BERG*,* 1995; GILANY *et al.,* 2008). Essas células apresentam características dopaminérgicas, pois expressam baixos níveis de enzimas da via de síntese das catecolaminas, incluindo a tirosina-hidroxilase (TH), dopamina-β-hidroxilase e transportador de dopamina (DAT) (XIE *et al.,* 2010).

A linhagem SH-SY5Y pode ser definida como neuroblastos imaturos que proliferam durante um grande período de tempo. Isso é uma grande desvantagem, pois os neurônios apresentam baixas taxas de proliferação (LUCHTMAN & SONG, 2010). Apesar da origem tumoral, muitos estudos mostraram que as células de neuroblastoma SH-SY5Y podem ser diferenciadas em neurônios dopaminérgicos através do tratamento com ácido retinóico (AR) (PÅHLMAN *et al.*, 1984). O AR é a forma biologicamente ativa da vitamina A necessária para o desenvolvimento normal, incluindo apoptose,

diferenciação e morfogênese de diversos órgãos e sistemas, como o sistema nervoso (BAIN *et al.,* 1995). O papel dessa neurotrofina na diferenciação neuronal é mediado através de receptores ao ácido retinóico (RAR) e receptores retinóides X (RXR) (MARK *et al.*, 2006). *In vitro*, o AR promove a diferenciação e inibe a divisão celular, bloqueando a fase G1/S do ciclo celular (MALIK *et al.,* 2000). Dessa forma, o AR regula a transição das células precursora para a célula diferenciada pós-mitótica (ROSS *et al.,* 2000), como ilustra a Figura 3.

Fig. 3: Diferenciação *in vitro* do neuroblastoma humano SH-SY5Y induzida por AR (Adaptada de Edsjö *et al.*, 2007). AR: ácido retinóico, SFB: soro fetal bovino.

A indução do processo de diferenciação ocorre pelo aumento da produção de noradrenalina, pelo aumento da expressão da enzima enolase e pela expressão de fatores de crescimento. Isso leva à formação de projeções citoplasmáticas (neuritos), induzindo as células a modificarem sua morfologia epitelial para uma forma estrelada.

Células SH-SY5Y diferenciadas com AR durante 7 dias são um modelo adequado de estudo de doenças que acometem neurônios, uma vez que elas passam a apresentar características morfológicas como formato estrelado com crescimento de neuritos alongados e características bioquímicas como expressar marcadores neuronais como TH, enolase-neurônio-específica (NSE) e proteína nuclear específica de neurônio (NeuN) com concomitante diminuição de marcadores de células não diferenciadas (LOPES *et al.*, 2010).

2. OBJETIVOS

2.1 OBJETIVO GERAL

Pesquisas têm mostrado como a exposição prolongada a altas taxas de glicose no sangue causam danos ao sistema nervoso e o estresse oxidativo parece ter um papel fundamental nesse processo (Kumar *et al*, 2009; Fioretto *et al*, 2009). Já está bastante evidenciado que há comprometimento da função celular devido à toxicidade das ER geradas pela hiperglicemia em pacientes com DM tipo I e II não controlada (Evans *et al*, 2002; Robertson *et al*, 2003; Nishikawa & Araki, 2007); no entanto, os fatores e mecanismos da glicotoxicidade nas células neurais permanecem desconhecidos, pois não se têm dado muita atenção a essa relação (Obrosova, 2009).

O objetivo deste estudo foi investigar os mecanismos celulares envolvidos com a neurotoxicidade das glicotoxinas. Utilizamos a linhagem diferenciada do neuroblastoma humano SH-SY5Y desafiadas com as glicotoxinas MG e GA. A sequência de eventos moleculares que leva a morte seletiva desses neurônios pela ação dessas glicotoxinas ainda não está estabelecida, portanto a identificação dos seus mecanismos de oxidação pode gerar novas oportunidades terapêuticas associadas ao combate à ND.

2.2 OBJETIVOS ESPECÍFICOS

- Realizar testes de brometo de metiltiazolildifenil-tetrazolium (MTT) para avaliar a viabilidade celular após tratamento com diferentes concentrações de MG e GA, determinando os respectivos valores de DL_{50} (dose da toxina que leva a uma perda de 50% da viabilidade celular) após 24 horas de exposição;

- Estimar a produção de ER das células diferenciadas tratadas com o DL_{50} das glicotoxinas através do teste de oxidação da sonda 2,7-diclorofluoresceína (DCF) em tempo real;

- Quantificar a variação no conteúdo de GSH total nos extratos celulares após 15, 45, 90, 180 e 360 minutos de exposição a dose correspondente ao DL_{50} do MG ou do GA;

- Caracterizar o tipo de morte celular (apoptose, necrose) que está sendo induzida pelo tratamento através da quantificação da lactato-desidrogenase (LDH) no sobrenadante, do ensaio de marcação com os compostos fluorescentes hoechst 33342 e iodeto de propídeo (IP) e da atividade das caspases 3, 8 e 9;

- Analisar a contribuição de diferentes sistemas antioxidades na detoxificação das glicotoxinas com a utilização de inibidores da tioredoxina-redutase (TrxR) e glutationa-redutase (GR), tratamento com doses subletais das toxinas e posterior avaliação da viabilidade celular com o teste de MTT.

PARTE II

3. RESULTADOS

Os principais resultados dessa dissertação estão apresentados na forma de artigo científico.

3.1 ARTIGO CIENTÍFICO

Manuscrito que será submetido para publicação no periódico

Neurotoxicity Research.

``OXIDATIVE STRESS AND PEROXIDASE SYSTEMS IN METHYLGLYOXAL AND GLYCOLALDEHYDE NEUROTOXICITY IN DIFFERENTIATED HUMAN SH-SY5Y NEUROBLASTOMA CELLS: INSIGHTS INTO THE PATOPHYSIOLOGY OF DIABETIC NEUROPATHY ``

OXIDATIVE STRESS AND PEROXIDASE SYSTEMS IN METHYLGLYOXAL AND GLYCOLALDEHYDE NEUROTOXICITY IN DIFFERENTIATED HUMAN SH-SY5Y NEUROBLASTOMA CELLS: INSIGHTS INTO THE PATOPHYSIOLOGY OF DIABETIC NEUROPATHY

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Abstract

In the nervous system, hyperglycemia causes damages to axons and myelin sheaths leading to impaired neuron function. Its known the accumulation of advanced glycation endproducts (AGE) is related to the pathogenesis of the diabetic neuropathy, but it is not well established what pathways are involved. Methylglyoxal (MG) and glycolaldehyde (GA) are reactive dicarbonyl compounds that are elevated under hyperglycemia conditions, they potentially induce oxidative stress and lead to cell growth arrest, apoptotic or necrosis death. These glycotoxins interact with sulfhydryl-groups of glutathione (GSH) molecules and enzymes, inhibiting their activity; however, the molecular mechanism underlying MG and GA cytotoxic effects are not fully understood. And, the contribution of antioxidant enzymes which catalyze the reduction of sulfhydryl groups like glutathione reductase (GR) and thioredoxin reductase (TrxR) in the neuronal defense against MG and GA cytotoxicities have not been investigated before. So, we have used the differentiated human neuroblastoma SH-SY5Y cells to evaluate the oxidative stress participation on the toxicity of these MG and GA and the role of GR and TrxR in the cells defense. In this study we show that MG and GA treatments lead to a fast increase in reactive species production with concomitant decrease in GSH content of the cells. Both MG and GA induce caspase-8 and -9 activation after 15 minutes of exposition, although just MG activate caspase-3, what happens after 90 minutes of exposition. Also, we reveal that TrxR has a most important role in cell defense against MG than GA, meanwhile there is no difference in the GR role. Our results show that oxidative stress is an important mechanism in the MG and GA toxicities and sulfhydryl reductors are very important in the cellular defense against these glycotoxins.

Keywords Diabetic Neuropathy ● Methylglyoxal ● Glycolaldehyde ● Oxidative Stress ● Neurotoxicity ● SH-SY5Y cells

Introduction

Long-term hyperglycemia leads to functional and structural deficits in both peripheral and central nervous system. Several studies have clearly identified hyperglycemia as a key feature involved in the pathogenesis of diabetic neuropathy (DN) (Tesfaye et al. 1996; Pop-Busui et al. 2010). In the nervous system, hyperglycemia is responsible for increased production of free radicals (Vincent et al. 2004; Edwards et al. 2008) that, together with a decreased neurotrophic support, damages axons and myelin sheaths (El Boghdady and Badr 2012).

The prevalence of neuropathy is estimated to be about 8% in newly diagnosed patients and greater than 50% in patients with long-standing disease (Boulton et al. 2005). Even though the pathogenesis of DN is likely multifactorial, investigations about the molecular and biochemical pathophysiology of DN have focused on glucose metabolic pathways that are related to the accumulation of AGEs (Ahmed 2005; Brownlee 2005; Huebschmann et al. 2006; Ahmed and Thornalley 2007), imbalance in the mitochondrial redox state of the cell (Zherebitskaya et al. 2009) and excess formation of reactive oxygen species (ROS) (Beisswenger et al. 2005; Fernyhough et al. 2010).

The accumulation of AGEs, was not only identified as a biologic phenomenon in the pathogenesis of DN, but also in the aging process and in degenerative conditions including cataracts and Alzheimer's disease (Amicarelli et al. 2003; Kikuchi et al. 1999; Shinpo et al. 2000). AGEs commonly arise from reaction of reducing sugars, such as glucose and short chain aldehydes, with amino groups (Ahmed 2005; Toth et al. 2008). The sugar moiety of an early glycation product can undergo chemical reactions, consequently producing low molecular weight carbonyls such as MG, glyoxal and GA (Thornalley 2005).

MG is inevitably produced in the course of metabolism even under normal conditions (Phillips & Thornalley 1993a; Richard 1991), by both enzymatic and non-enzymatic reactions, but the major source of MG is the nonenzymatic fragmentation of triosephosphates (Phillips & Thornalley 1993b). GA is a short-chain aldehyde formed as a by-product of protein glycation and also by the myeloperoxidase activity upon amino acids (Tobler and Koeffler 1991; Al-Enezi et al. 2006). These oxyaldehydes contribute significantly to intracellular AGE formation, since they are extremely reactive (Glomb and Monnier 1995; Turk 2010; Wang and Ho 2012). They can interact with the SH-groups of proteins, inhibiting the activity of several enzymes (including the antioxidant ones) and critical cellular proteins that can potentially lead to cell growth arrest, apoptotic or necrotic death (Ramasamy et al. 2006). However, the molecular mechanisms underlying this cytotoxic effect are not yet fully understood.

MG forms a hemithioacetal adduct with GSH, which plays an important role in the protection of cells against oxidative damage (Di Loreto et al. 2004; Di Loreto et al. 2008; Kalapos et al. 2008). Furthermore, MG is mainly detoxified by the GSH-dependent glyoxalase system which may lead to cellular GSH depletion (Thornalley 2003). Together, both the depletion of intracellular GSH and the inhibition of several antioxidant defense enzymes make cells more susceptible to oxidative stress (Di Loreto et al. 2008; Kalapos et al. 2008). Thus, enzymes that can act as sulfhydryl group reductases must be important in the defense against MG and GA toxicities. GR is an enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH; and TrxR is the enzyme that catalyzes the reduction of thioredoxins - proteins with important thiol-specific antioxidant role (Ácimovic et al. 2010). However, the contribution of these antioxidant enzymes on neuron cells defense against glycotoxins was never evaluated before.

There are many studies evaluating the cytotoxic features of glycotoxins, but there is little information regarding their effects over the central nervous system and neuronal cells models, such as differentiated human neuroblastoma SH-SY5Y cells. Thus, we used the differentiated human SH-SY5Y neuroblastoma cell line to explore the mechanisms underlying MG and GA induced neurotoxic effects and the contribution of TrxR and GR antioxidant systems in the defense against this toxins. This experimental model is well characterized and has been used as an *in vitro* system to investigate the pathological effects of various neurotoxic compounds (Lopes et al. 2010; Lopes et al. 2012). In addition, SH-SY5Y cells constitutively express a receptor for AGEs and they also are highly sensitive to MG challenge (García de Arriba et al. 2007; Li et al. 2011).

Experimental Procedures

Chemicals

Materials used in cell culture were acquired from Gibco/Invitrogen (São Paulo, SP Brazil). Other reagents and solvents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell Culture and Neuronal Differentiation

Exponential growing human neuroblastoma cell line SH-SY5Y, obtained from ATCC (Manassas, VA, USA), were maintained in a mixture 1:1 of Ham's F12 and Dulbeco Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 10000 units/mL of penicillin, 10000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B in a humidified atmosphere of 5% of CO2 in air at 37ºC. Cell medium were replaced every three days and cell were sub-cultured once they reached 80% confluence. After 24 hours of cell plating, differentiation was induced by lowering the FBS in culture medium to 1% plus the addition of 10 µM retinoic acid (RA) during 7 days. This treatment was replaced each 3 days to replenish RA in culture media, as previously described (Lopes et al., 2010). All treatments were performed when cells were ~75% confluence. For the MTT, LDH, DCF and peroxidases inhibition assays the cells were plated in a 24-well plate at density of 7 x 10^4 cells per well. For caspase activity; GSH, GR and TrxR quantification cells were seeded into bottles of 75 cm^2 at density of 5 x 10^6 cells.

Cell Viability Assay

Cell viability was assessed by MTT method. MG and GA solutions were freshly prepared in cell culture medium. Drug cytotoxicities were evaluated by exposing cells to different concentrations of these glycotoxins for 24 hours at 37ºC, and cell viability was estimated by the quantification of the **3**-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction to a blue formazan product by cellular dehydrogenases. At the end of the treatments, the medium was discarded and the cells were washed with phosphate buffered saline (PBS). A new medium containing 0.5 mg/ mL of MTT was added. These cells were incubated for 1 hour at 37ºC. This medium was discarded and DMSO was added to solubilize the formazan crystals for 30 minutes. Absorbance was determined at 560 nm and 630 nm in a SoftMax Pro Microplate Reader (Molecular Devices®, USA). Viability values were expressed as percentage of control.

Measurement of Reactive Species Generation

The cellular reactive species was quantified using DCF assay. 2,7-dichlorofluorescein diacetate (DCF) 1,1 mM solution was made freshly in PBS from a stock solution (30 mM) made in DMSO. The solution was added to the cells in medium to a final concentration of 100 μM, and the plates were incubated for 1 hour in an atmosphere of 5% CO2 at 37ºC to load cells with DCF. Then, the medium was discarded and a new medium with the treatments was added. DCF was excited at 485 nm, and fluorescence emission at 538 nm was recorded using a SoftMax Pro fluorescence plate reader (Molecular Devices®, USA).

Total GSH Cellular Content

Total GSH (t-GSH) content was measured using a assay based in the GSH recycling system by 5,5-dithio-(2-nitrobenzoic acid) (DTNB). DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid and GSSG. Then, GSH is regenerated from GSSG by GR and react with DTNB again to produce more 2-nitro-5-thiobenzoic acid. Therefore, the concentration of GSH was determined by measuring absorbance of 2-nitro-5-thiobenzoic acid at 412 nm. Cells were treated with MG or GA LD_{50} for 15, 45, 90, 180 and 360 minutes. Then, were scraped out from de culture flask, collected by centrifugation at 1000 g for 5 min and the supernatant was discarded. The pellet was washed with PBS and centrifugated again. The supernatant was discarded and the cells were resuspended in Hepes 20 mM. CHAPS was added to a final concentration of 1 % and cells were incubated for 30 minutes in ice. The lysates were centrifuged at 1000 g for 5 minutes and the supernatants were collected. The protein content was measured. Proteins were precipitated with perchloric acid (PCA) in a final concentration of 0,5 M. Centrifugation was performed at 15000 g for 5 min and supernatant was collected. 30 μL of each sample was diluted in 0,1 M K_2HPO_4, 1 mM EDTA buffer for neutralization. In a 96 wells plate were pipetted 37,5 μL of neutralized samples, 200 μL of assay buffer (K_2HPO_4 0,25 M, EDTA 2,5 mM, NADPH 100 mM, DTNB 5 mM), 12,5 μL of GR (5 U/mL) and the assay was performed using SoftMax Pro plate reader (Molecular Devices®, USA) at 412 nm for 5 minutes.

Hoechst 33342 and Propidium Iodide dual-labeled dyeing

To analyse the occurrence of apoptosis/necrosis events induced by the LD_{50} doses of MG or GA after 24 h, 1μg/mL per well of the fluorescent dye Hoechst 33342 was added and incubated for 15 minutes in a humidified atmosphere of 5% of CO2 in air at 37ºC. Then, the medium was descarted, cells were whashed with PBS and pictures were obteined in a fluorescence microscope. Propidium iodide (PI) was added imediately before the picture capture in a final concentration of 25 μg/mL. Fields of cells were photographed by using appropriate filters to examine Hoechst 33342 and PI fluorescence staining.

Lactate Dehydrogenase Activity

For determination of lactate dehydrogenase (LDH) released from cells, the culture medium was collected after a 24 h incubation period with LD_{50} doses of MG or GA and placed on ice until be used. LDH was measured by a LDH assay kit from Labtest®, following manufacturer's instructions. LDH release was calculated and expressed as a percentage of the LDH measured in untreated cells (control).

Caspase-3, -8 and -9 Activities

Caspase-3, -8 and -9 activities were determined by the cleavage of the fluorescent substrates Ac-DEVD-AFC, Ac-IETD-AFC and Ac-LEHD-AFC respectively. Cells were treated with the MG or GA LD_{50} for 15, 45, 90, 180 and 360 minutes. Then, were scraped out from de culture flask, collected by centrifugation at 1000 g for 5 min and the supernatant was discarded. The pellet was washed with PBS and centrifuged again. The supernatant was discarded and the cells were resuspended in lyses buffer (50 mM HEPES, 100 mM NaCl, 1 mM DTT, 100 mM EDTA, pH 7.4). CHAPS was added to a final concentration of 0,2 %. After that, cells were frozen at -80^{o}C and thawed three times. Cells extracts were centrifuged at 14000 g for 15 minutes and the supernatant was collected. The protein content was measured. In a 96 wells black plate, were pipetted 20 μg of protein per well and assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 mM EDTA , 10% glycerol, pH 7.4) to a final volume of 100 μL, then fluorescent caspase substrate 3, 8 or 9 were added to a final concentration of 50 μM. The plate was incubated at 37^{o}C for 2 hours before reading. Samples were assayed using SoftMax Pro fluorescence plate reader (Molecular Devices®, USA).

Inhibition Studies

Differentiated SH-SY5Y cells were pretreated for 30 minutes with acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylthiocarbonylamino)phenylthiocarbamoylsulfanyl]propionic acid hydrate S,S′-[1,4-Phenylenebis(iminocarbonothioyl)]bis[N-acetyl-L-Cysteine] hydrate) (2-AAPA), or auranofin (AU) - inhibitors of GR and TrxR, respectively - and the specific enzymatic activity and cell viability was measured. A non lethal dose of each drug, able to inhibit their respective enzymes activities, was established. The contribution of each enzymatic system in MG and GA toxicity was evaluated, the differentiated cells were pretreated for 30 min with the inhibitors (10 μM of 2-AAPA or 0,5 μM of AU) and exposed to sublethal doses of MG (625 μM) or GA (25 μM) for 24 hours. Cell viability was determinated by MTT assay.

Antioxidant Enzyme Assays

For antioxidant enzyme assays, the differentiated SH-SY5Y cells were homogenized in Hepes 20 mM, pH 7.0, and centrifuged at 15,000 g for 30 minutes at 4 °C. Enzyme activities were then determined in the supernatant according to standard methods (Arnér et al. 1999; Carlberg and Mannervik 1985) for GR, or TrxR activity, respectively. Enzyme activities were expressed as a percentage of untreated samples.

Protein Quantification

The proteins contents were measured by the Bradford assay (Bradford, 1976), using bovine serum albumin as standard.

Statistical Analysis

All data were expressed as mean ± SD. GraphPad Prism 5 was used to determine statistical significance. Difference between two groups was analyzed by two-tailed Student's *t* test, difference among three or more groups was analyzed by two-way anova and differences among different treatments times were analyzed by repeated measures ANOVA. $P < 0.05$ was considered statistically significant.

Results

Determination of Methylglyoxal and Glycolaldehyde LD_{50} in Differentiated SH-SH5Y Cells

To evaluate the MG and GA toxicities in differentiated SH-SY5Y cells and in order to establish a standard concentration of these glycotoxins for this study, we set the concentration of each toxin able to decrease fifth percent of cells viability (LD_{50}). Cells were treated with different doses of MG or GA for 24 hours and the toxicity was evaluated using the MTT assay. A dose response curve was obtained and a dose dependent cell death can be observed (Fig. 1a and 1b). The LD_{50} in differentiated SH-SH5Y cells was established as 1350±16 μM for MG and as 115±3.37 μM for GA for 24 hours of exposition.

MG and GA Treatment Led to an Increase in Reactive Species (RS) Production

In order to investigate if oxidative stress is involved in MG and GA cytotoxycities we performed a real time DCF oxidation assay in treated cells. The increase in DCF fluorescence is a current method to measure the production of intracellular RS in neurons. Time course experiment was performed using the DCF dye to evaluate the generation of reactive species by glycotoxins (Fig. 2a). In the first six hours of treatment with LD_{50} value of GA was detected a fast increase in DCF fluorescence detection when compared to control. Treatment with MG LD_{50} value has not caused a significant RS increase in the first six hours; however, both glycotoxins have increased RS generation after 24 hours of exposition (Fig. 2b).

MG and GA Treatments Caused a Fast Decrease in t-GSH Content

Aiming to corroborate with the involvement of oxidative stress in MG and GA neuronal citotoxycities, we evaluated the total GSH cell content in treated cells. T-GSH content significantly decreased immediately after both treatments with LD_{50} values, but rapidly recovered to almost normal levels in GA treated cells. MG caused a more accentuated and lasting reduction in t-GSH content (Fig. 2c).

Apoptosis is the Main Cell Death Mode Induced by MG and GA Treatments

The cells were stained with color combination of DNA-specific dyes Hoechst 33342 and PI. Control cells demonstrated an intact structure, they excluded PI and stained mostly with Hoechst in blue color, indicative of cell membrane integrity. In the occurrence of apoptosis the nuclear morphology was disrupted and those cells were more brightly stained indicative of chromatin condensation in globular or crescent-shaped figures. In necrosis was observed the staining with PI in red color, indicative of loss of membrane integrity. Also, was noticed the occurrence of late apoptosis with chromatin condensation and PI staining. Cells treated with MG demonstrated the occurrence of the three phenomenoms; however apoptosis is the more prevalent one. In cells treated with GA only apoptosis was observed (Fig. 3a).

MG and GA Treatments Caused a Slight Increase in LDH Release

The membrane integrity of the cells was evaluated by measuring the release of intracellular LDH into the cultura medium. This enzyme converts pyruvate to lactate by consuming NADH, which is kinetically measured in the supernatant, which directly correlates to the amount of LDH released, which is used as a necrotic index. MG and GA led to a slight increase in the amount of LDH released, especially in MG-treated cells (Fig. 3b).

MG and GA Activate Caspase-3, -8 and -9

To test if apoptosis was responsible for the death of differentiated SH-SH5Y cells observed after MG and GA treatments, we investigated caspases-3, -8 and -9 activities in these cells using a synthetic substrate that contains the cleavage site recognized by these proteases. A time curve among 15 minutes and 6 hours had been performed in order to determine the kinetics of caspase activation. MG caused a 3,5 fold increase in caspase-8 activity and ≈ 2,5 fold increase in caspase-3 and -9 (Fig. 3c). GA caused ≈ 2,5 fold increase in caspase-8 and -9 activities and ≈ 1,4 fold increase in caspase-3 (Fig. 3d). After 15 minutes of exposition to the glycotoxins both caspase-8 and -9 are activated. MG treatment has led to caspase-3 activation after 90 minutes and GA treatment did not cause significant activation of this caspase.

Inhibition of GR and TrxR Improve MG and GA Toxicities

To evaluate the contribution of different peroxidases systems in the defense of differentiated SH-SY5Y cells against MG and GA cytotoxicities we inhibited GR and TrxR activities, challenged the cells with sublethal doses of these glycotoxins and evaluated the cell viability after 24 hours. For this purpose, we first established the best conditions to specifically inhibit each peroxidase systems and to determine the toxicities of these inhibitors (Fig. 4a, b and c). The pretreatment of differentiated SH-SH5Y cells for 30 min with inhibitors of GR (2-AAPA) and TrxR (AU) decreased the corresponding activity by 10–85%, depending on the enzyme and concentration of the inhibitor (Figs. 4a and b). Fig. 4c shows that cell viability was significantly decreased by 2-AAPA doses higher than 10 μM and AU doses higher than 0,5 μM, so these doses were established for the inhibition induction. The pretreatment with AU contributed to a decrease in cell viability of 31% in MG treatment and of 15% in GA treatment, suggesting that TrxR system is more important in the detoxification of MG than GA (Fig. 4e and g). The pretreatment with 2-AAPA contributed to a decrease in cell viability of 25% in both treatments, not showing difference of importance in the detoxification of these glycotoxins (Fig. 4d and f).

Discussion

The pathological process of diabetic neuropathy is complex, involving many different factors like glucose flux through the polyol pathway (Yagihashi et al. 2001; Obrosova et al. 2002); excess/inappropriate activation of protein kinase C (PKC) isoforms (Price et al. 2004); poly (ADP-ribose) polymerase (PARP) overactivation (Li et al. 2005; Obrosova et al. 2008), accumulation of AGE (Tanji et al. 2000), oxidative/nitrosative stress (Cameron et al. 2001; Coppey et al. 2001; Obrosova et al. 2001) and mitochondrial dysfunction (Zhang et al. 2012). Reactive carbonyl compounds, which are either products of glucose metabolism or oxidative damage to lipids, contribute to GSH depletion and antioxidant enzymes inactivation, leading to a toxic burden in already stressed neurons (Kalapos et al. 2008). In particular, dicarbonyl compounds such as MG and GA have been suggested as neurotoxic mediators of oxidative damage in the progression of diabetic's complications, neurodegenerative diseases and even in aging processes (García de Arriba et al. 2006). In addition, diabetic patients showed plasma MG

concentrations of about 400 μM (Lapolla et al. 2003b). As well, the exposure of rats to increased MG doses (50–75 mg/kg) *in vivo* was associated with the onset of microvascular damages and other diabetic-like complications within a normoglycemic context (Berlanga et al. 2005). Cytotoxicity of MG and glicolaldehyde has already been reported for fibroblasts, macrophages, insulin-secreting cells, hepatocytes, neurons and many others mammalian cells (Amicarelli et al. 2003; Al-Enezi et al. 2005; Wang et al. 2009; Yang et al. 2011; Koizumi et al. 2011). It has been shown that MG inhibits several key enzymes, such as ATPases (Mira et al. 1991; Derham et al. 2003), glyceraldehyde 3-phosphate dehydrogenase (Halder et al. 1993), complex I of the mitochondrial respiratory chain (Biswas et al. 1997; Rosca et al. 2002), glutathione reductase, and lactate dehydrogenase (Morgan et al. 2002).

Some recent studies have shown that SH-SY5Y neuroblastoma cells show greater sensitivity to MG challenge due to a defective antioxidant and detoxifying ability (Lee et al. 2012; Li et al. 2011; García de Arriba et al. 2007; Kuhla et al. 2006), but there is no study investigating GA toxicity in SH-SY5Y cells. In primary neuron culture, it was demonstrated that GA can affect neuronal survival, neurite density and cell morphology (Luo et al. 2002). However, there isn't any study involving glycotoxicity in differentiated SH-SY5Y neuroblatoma cells, even though 7-day-RA-differentiated form of SH-SY5Y cells represents a more suitable experimental model for studying the molecular and cellular mechanisms underlying the pathophysiology of neurodegenerative processes. This experimental model has a neuronal morphology and express several neuronal markers such as tyrosine hydroxylase, neuron specific enolase (NSE) and neuronal nuclei protein (NeuN) (Lopes et al. 2010). So the main goal of this study was to understand the molecular mechanism underlying the responses to MG and GA toxicities, using the differentiated SH-SY5Y cells, and establish the contribution of several peroxidase systems in drug detoxification.

In the present study we clearly demonstrated that MG and GA treatments have cytotoxic effects on differentiated SH-SY5Y cells by inducing oxidative stress and apoptotic death. MG and GA cytotoxicities in differentiated SH-SY5Y cells were dose and time dependent (data not shown). It is known that in neurons high concentrations of MG and long incubation periods (up to 24 h) are required to induce cytotoxicity (Shangari and O'Brien 2004; Shinpo et al. 2000; Kikuchi et al. 1999); this is in accordance to our findings where a dose of 1350 μM of MG is necessary to kill fifth percent of the cells in 24 hours of treatment. Otherwise, GA seems to be more toxic, probably due to its high chemical reactivity to amino residues of proteins, so much that the direct determination of GA is practically impossible (Glomb and Monnier 1995).

It is already stated that MG accumulation is responsible for detrimental effects on neurons viability through the induction of reactive oxygen species (ROS) leading to oxidative damage mediated cell death (Di Loreto et al. 2004; Koizumi et al. 2011). Studies from antioxidant capacity have been demonstrated that the susceptibility of neuronal cells to MG is mainly due to a strong weakening of antioxidant systems, consequence of the impairment in catalase, SOD and GPx activities and the strong depletion in intracellular GSH (Di Loreto et al. 2004; Di Loreto et al. 2008). There are some reports of oxidative stress involvement in GA toxicity too. Increases of superoxide radical production, lipid peroxidation, and accumulation of protein carbonyl had already been described as consequences of GA treatment (Al-Enezi et al. 2005; Lorenzi et al. 2010a; Lorenzi et al. 2010b).

In this study, MG and GA cytotoxicities were preceded by a depletion of GSH levels and increased RS production, both events occurred in the first 90 minutes of exposition to the glycotoxins. The extensive and early RS production found after MG and GA treatments suggests that oxidative stress may be a major contributor to MG and GA toxicities and, that RS might act as primary factors in the cell signaling pathway triggered by MG and GA, as already suggested for other cell types (Du et al. 2001; Okado et al. 1996). This fast redox imbalance has been reported by others researches (Amicarelli et al. 2003; Garcia de Arriba et al. 2006; Di Loreto et al. 2008; Okouchi et al. 2009). Although after 6 hours of treatment the GSH levels are almost restored to untreated levels, high RS generation was maintained after 24 hours. This may be consequence of protein and lipid glycation by MG and GA, producing AGEs that feed the oxidative process. MG has induced a greater and long lasting decrease in GSH levels than GA, and a possible explanation to this could be the detoxifying system involved with each toxin. MG is enzymatically metabolized by the glyoxalase system (Thornalley 1996; Thornalley 2003), which consists of two enzymes: glyoxalase I and glyoxalase II. This system uses GSH as a cosubstrate to convert MG to the harmless compound D-lactate. High doses of MG could over-activate this system, leading to GSH depletion. Nevertheless, the mechanism of GA detoxification and its metabolic fate remained largely unknown, however a recent study showed that the alcohol dehydrogenase, which is capable of reducing short-chain aldehydes such as acetaldehyde and formaldehyde, can convert GA to ethylene glycol by using NADH as cofactor (Jayakody et al. 2012). Taken together the result of the decreased GSH levels and the increased RS production on cells exposure to MG and GA, oxidative stress appears to be involved

with the mechanisms of cytotoxicity of these glycotoxins, and its seems to be an early event on the death process.

To better investigate this, we evaluated the activation status of caspases-3, -8 and -9; all they had previously been related to MG or GA toxicity (Amicarelli et al. 2003; Okouchi et al. 2009; Moheimani et al. 2010). Our Hoechst 33342 and PI staining image data show that apoptosis is the main type of death triggered by MG and GA, and the massive caspase activation corroborate to that. Treated differentiated SH-SY5Y undergoes programmed cell death through both extrinsic and intrinsic pathways, demonstrated by the activation of caspases-8 and -9, respectively. Previous data reports that MG-mediated apoptosis occurs via the intrinsic pathway, typically triggered by oxidative stress, growth factor withdrawal, chemotherapeutic agents and irradiation (Amicarelli et al. 2003; Okouchi et al. 2009). Moreover, the MG activation of the extrinsic pathway had already been reported and supported by the increase of proinflammatory cytokines and TNF-α, well known caspase-8 regulator (Di Loreto et al. 2008). Actually, it seems that the treatment of differentiated SH-SY5Y cells with MG and GA induces apoptosis through the activation of both routes; the mitochondrial, since it was well established that MG and GA induce oxidative cell injury. Furthermore, we found that MG treatment activates caspase-3 after 90 minutes of exposition. This quite early activation could explain why we found cells that have condensed chromatin and also PI incorporation; it could explain either the increase in LDH release, once LDH release had been quantified only 24 hours after treatment. If apoptosis occurred promptly, the apoptotic bodies could be deteriorated at this time - event called late apoptosis (Poon et al. 2010). GA treatment had not been detected significant caspase-3 activation, probably because it would happen latter and only the first six hours of exposition have been analyzed.

Several studies show that different cell lines have diverse sensitivities to MG and GA, this could be ascribed to cell-type-specific differences in the capacity to counteract the adverse effects of RS. Due to the fact that undifferentiated and differentiated SH-SY5Y cells don't have the same redox profile (Lopes et al, unpublished results) this study brings novel data over MG and GA toxicities. In order to further study this aspect, we evaluated the contribution of antioxidants systems in the metabolisms of MG and GA. It was performed by the TrxR and GR inhibitions since cellular protection against oxidative damage of proteins involves two this thiol-disulfide oxidoreductase systems - the thioredoxin (Trx) and glutaredoxin (Grx) systems (Berndt et al. 2007). The inhibition was followed by an exposition to sublethal doses of these glycotoxins. Our data suggest the TrxR has a more important role in cellular defense against MG than against GA, because the inhibition of TrxR has increased cellular death in 31% in the MG essays but in the GA essays this increase was of only 15%. Meanwhile, GR inhibition seems to have the same importance in MG and GA death mechanisms, since it has potentiated the lost of viability in 25% in both glycotoxins essays.

Our results show that MG and GA treatments have led to a fast decrease in cellular GSH content, increased RS production and early caspase activation. Taken together, our findings support the idea that MG and GA treatments shift the cellular environment towards a more oxidized state. Also, we can say that MG and GA have distinct detoxification processes in differentiated SH-SY5Y cells, and our results may provide some novel insights into the molecular mechanisms underlying these glycotoxins induced neurodegeneration.

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FIG. 1: MG and GA viability curves in differentiated human neuroblastoma cell line SH-SY5Y. Cells were treated with different concentrations of **(a)** methylglyoxal or **(b)** glycolaldehyde for 24 h, and the cytotoxicities were evaluated using the MTT assay, as described in experimental procedures section. Results were calculated as a percentage control (untreated) cells. LD_{50} values were obtained for both glicotoxins. The data represent means±SD for three experiments carried out in quadruplicates.

FIG. 2: MG and GA redox consequences in differentiated human neuroblastoma cell line SH-SY5Y. To reactive species generation assess, cells charged with DCFDA were treated with LD_{50} values of ■ methylglyoxal and ▼ glycolaldehyde, than DCF fluorescence was assayed as described in experimental procedures section. Fluorescence was measured **(a)** during the first 6 hours and **(b)** after 24 hours of glycotoxins treatments. Different from untreated by repeated measures ANOVA **P*<0,05; ***P*<0,01; ****P*<0,001. **(c)** To time curve measurement of total glutathione, cells were treated with LD_{50} values of ● methylglyoxal and ▼ glycolaldehyde, and t-GSH was assayed as described in experimental procedures section. Measurements of total GSH were performed after 15, 45, 90, 180 and 360 minutes of glycotoxins treatments. Different from untreated by repeated measures ANOVA ****P*<0,001 The data represent means±SD for three experiments carried out in quadruplicates.

FIG. 3: Neuroblastoma cell line SH-SY5Y death induction by MG and GA. (a) Fluorescence imaging for determination of cell death mode induced by the MG and GA LD_{50} values. The cells were treated for 24 hours and then stained with the Hoechst 33342 and PI. The first line shows the contrast phase, the second line shows the Hoechst 33342 staining and the third line shows the PI staining of the corresponding columns: left, untreated cells; middle, MG treated cells; and right, GA treated cells. The white arrows show apoptosis, the light gray arrows show necrosis and the dark gray arrows show late apoptosis. The images are representative of three experiments carried out in quadruplicates. **(b)** To evaluate LDH release, cells were treated with LD_{50} values of methylglyoxal and glycolaldehyde and after 24 hours supernatant medium was collected. LDH release was assayed as described in experimental procedures section. Different from untreated by one-way ANOVA ****P*<0,0001. To time curve measurement of caspase-3, -8 and -9 activation, cells were treated with LD_{50} values of **(c)** methylglyoxal and **(d)** glycolaldehyde, and caspases-3, -8 and -9 activation was assayed as described in experimental procedures section. Measurements were performed after 15, 45, 90, 180 and 360 minutes of glycotoxins exposition. Different from untreated by repeated measures ANOVA **P*<0,05; ****P*<0,001. The data represent means ± SD for three independent experiments.

FIG. 4: Antioxidants systems contribution in defense against MG and GA toxicities in differentiated human neuroblastoma cell line SH-SY5Y. Cells were pretreated for 30 minutes with Au or AAPA, the TrxR and GR inhibitors, respectively. The specific enzymatic activity of **(a)** TrxR and **(b)** GR were measured. And, **(c)** the cell viability was assayed. Different from untreatment by repeated measures ANOVA **P*<0,01; ***P*<0,001; ****P*<0,0005. Then, cells pretreated for 30 minutes with **(d)** AAPA or **(e)** Au were exposed to sublethal doses of methylglyoxal (625 μM). And cells pretreated for 30 minutes with **(f)** AAPA or **(g)** Au were exposed to sublethal doses of glycolaldehyde (25 μM) for 24 hours, and cell viability was assayed. Different from control treatment by two-way ANOVA ****P*<0,0005. Results were calculated as a percentage of values obtained for control cells. The data represent mean ±SD for three experiments carried out in quadruplicates.

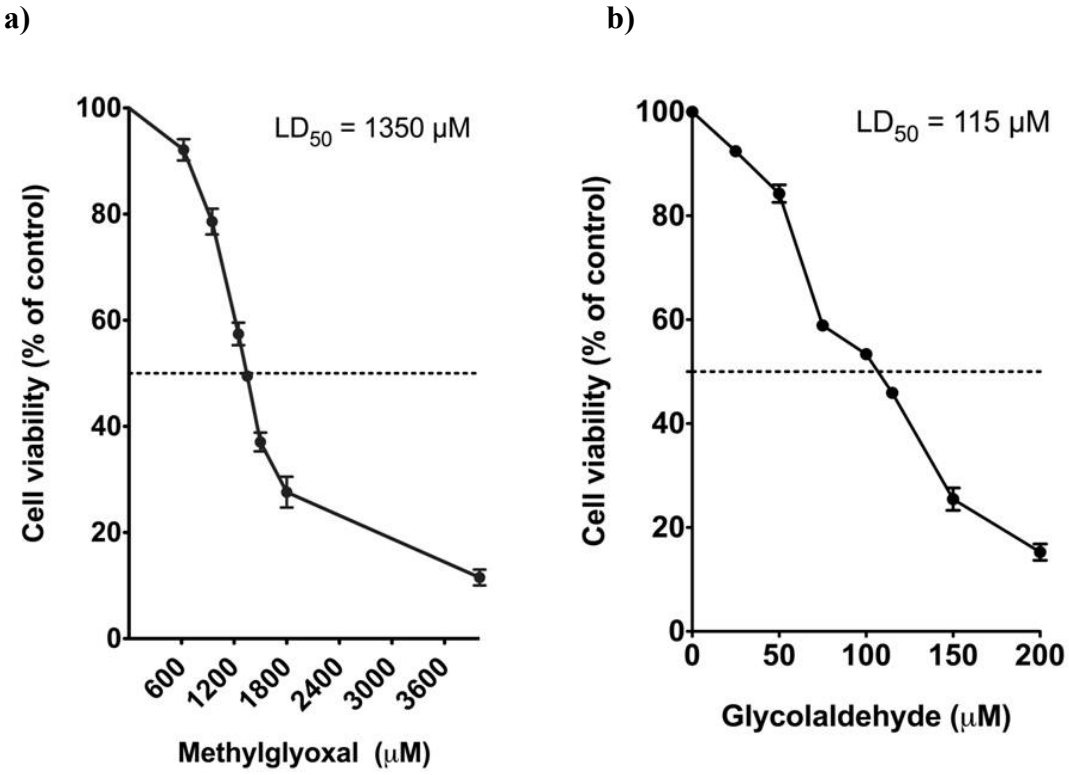

a)
b)
LD50 = 1350 µM
LD50 = 115 µM
Cell viability (% of control)
Methylglyoxal (µM)
Glycolaldehyde (µM)
0
20
40
60
80
100
600
1200
1800
2400
3000
3600
0
50
100
150
200

FIGURE 1

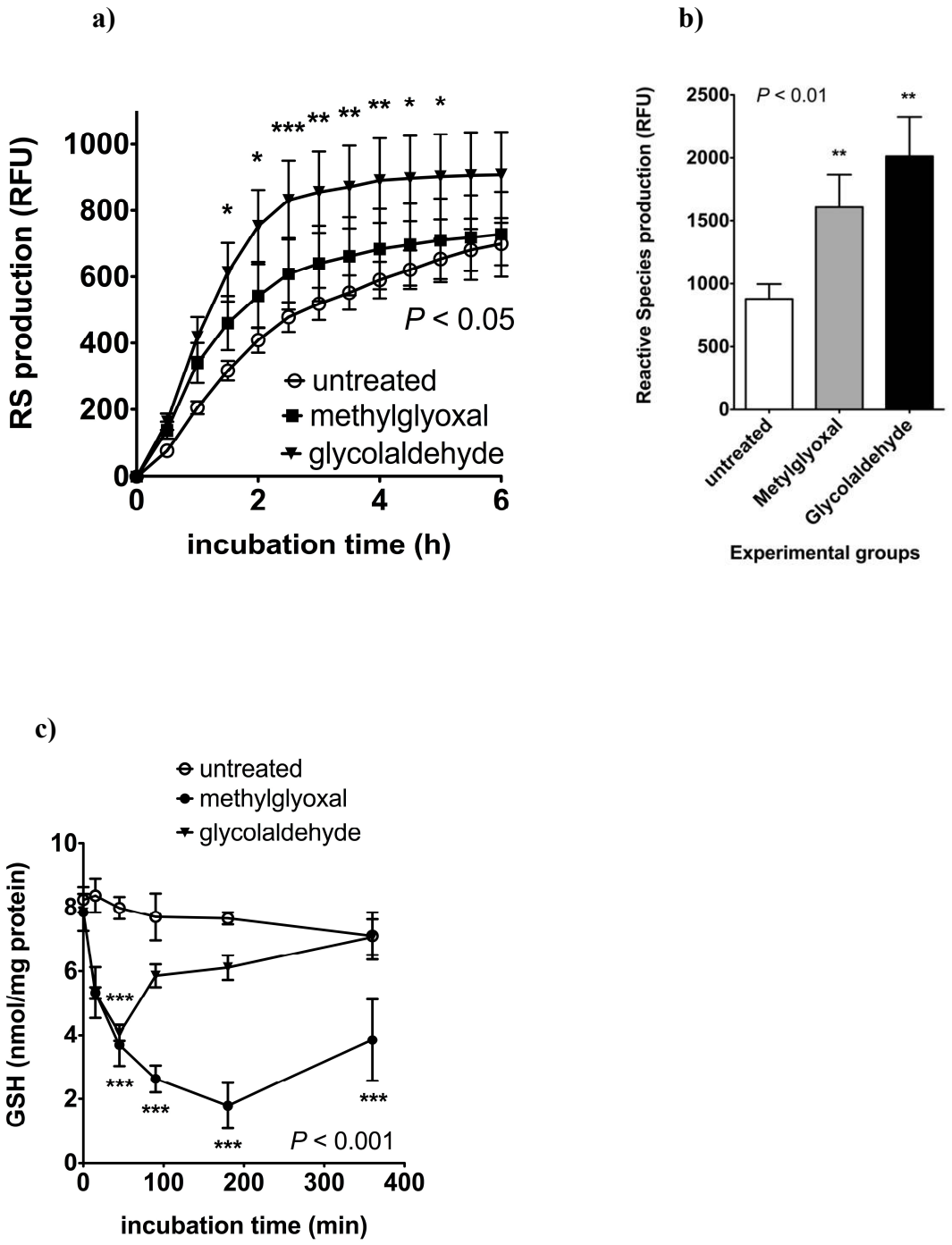
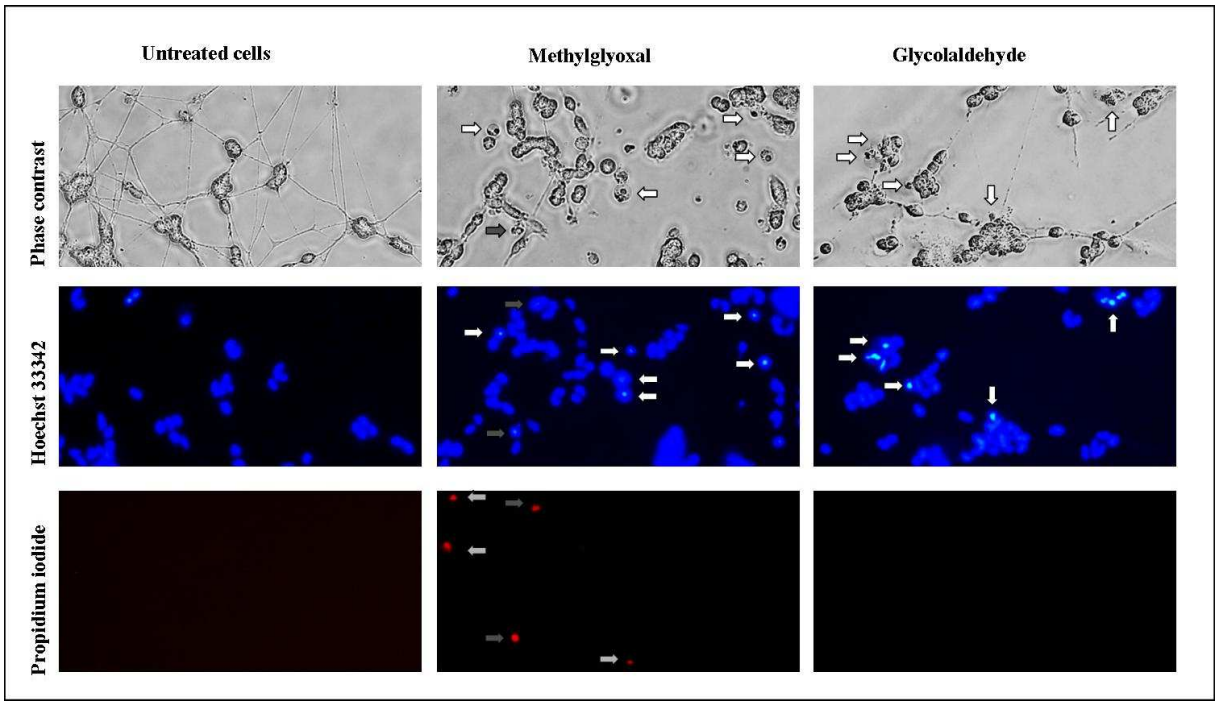
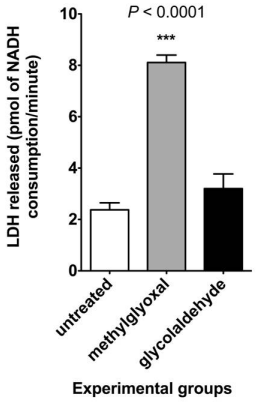

a)
RS production (RFU)
1000
800
600
400
200
0
0
2
4
6
incubation time (h)
P < 0.05
untreated
methylglyoxal
glycolaldehyde
b)
Reactive Species production (RFU)
2500
2000
1500
1000
500
0
P < 0.01
untreated
Metylglyoxal
Glycolaldehyde
Experimental groups
c)
untreated
methylglyoxal
glycolaldehyde
GSH (nmol/mg protein)
10
8
6
4
2
0
0
100
200
300
400
incubation time (min)
P < 0.001

FIGURE 2

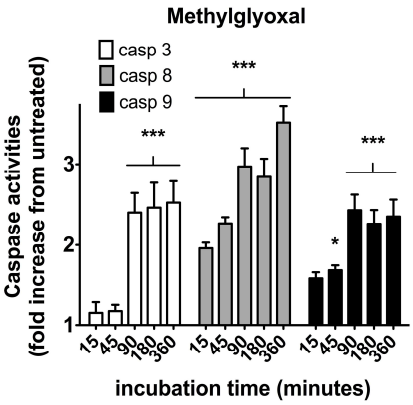
a)



b)



c)



d)

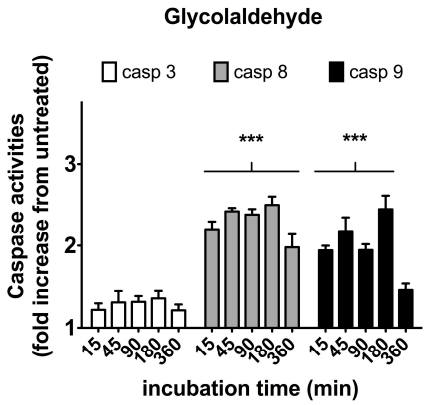


FIGURE 3

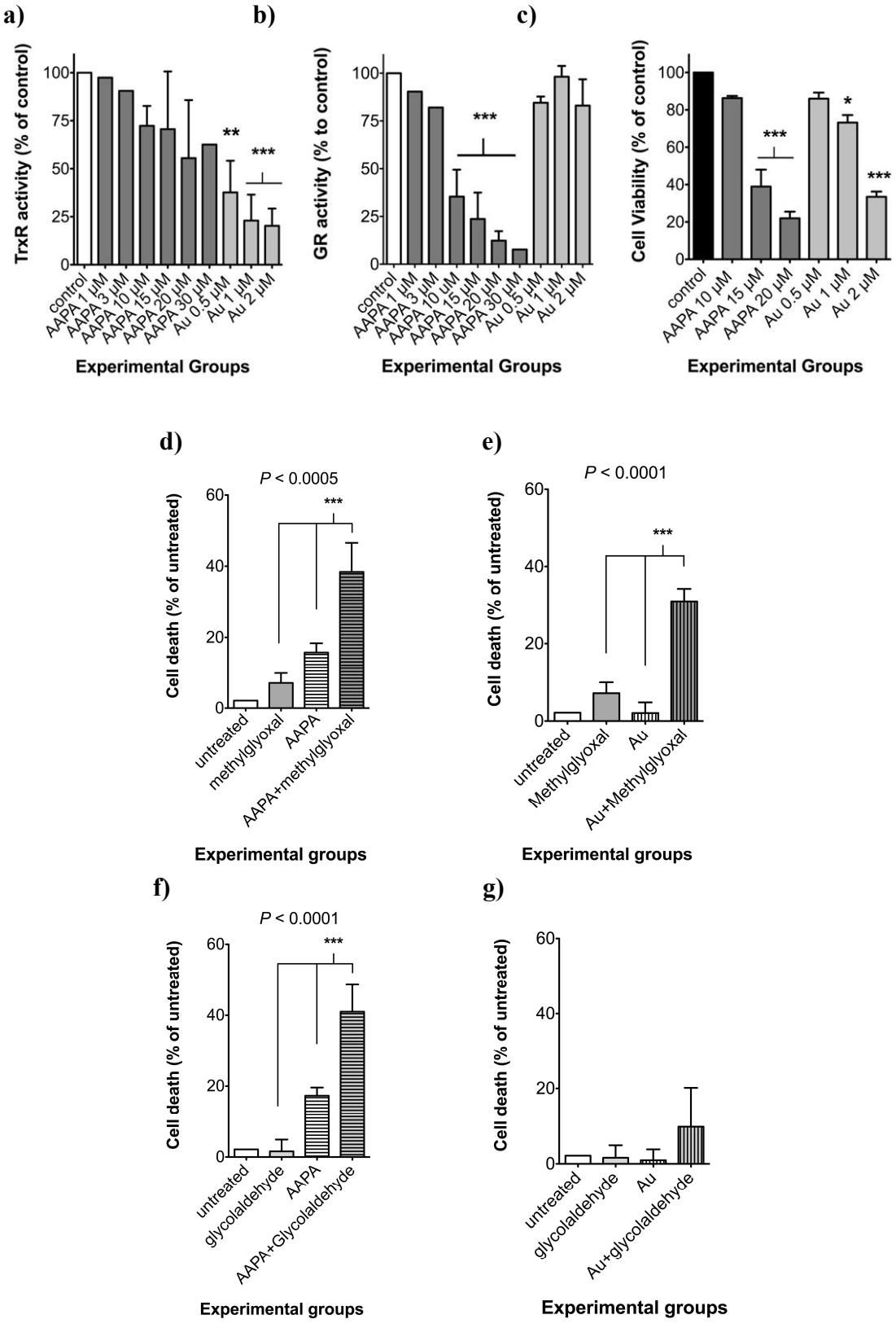

a)
b)
c)
TrxR activity (% of control)
GR activity (% to control)
Cell Viability (% of control)
control
AAPA 1 µM
AAPA 3 µM
AAPA 10 µM
AAPA 15 µM
AAPA 20 µM
AAPA 30 µM
Au 0.5 µM
Au 1 µM
Au 2 µM
Experimental Groups
d)
e)
f)
g)
P < 0.0005
P < 0.0001
Cell death (% of untreated)
untreated
methylglyoxal
AAPA
AAPA+methylglyoxal
Methylglyoxal
Au
Au+Methylglyoxal
glycolaldehyde
AAPA+Glycolaldehyde
Au+glycolaldehyde
Experimental groups

FIGURE 4

PARTE III

4. DISCUSSÃO

A neuropatia é uma complicação comum tanto da DM tipo 1, como da DM tipo 2. A prevalência da ND é de cerca de 8% em pacientes recentemente diagnosticados e, maior do que 50% em pacientes que já têm a doença há mais de 25 anos (BOULTON *et al*., 2005). Além disso, existem evidências de que mesmo em um estágio de pré-diabetes já existe a ocorrência da ND (FRANKLIN *et al*., 1990; SINGLETON *et al*., 2003). Essa complicação da *diabetes* DM é a principal causa da amputação de membros (THOMAS, 1999); e, seus custos anuais chegam a 11 bilhões de dólares nos EUA (GORDOIS *et al*., 2003), segundo país com maior ocorrência dessa doença (INTERNATIONAL DIABETES FEDERATION, 2008). O processo patológico da ND é complexo, e já foram descritos o envolvimento de diversos mecanismos como: superativação da via do poliol (YAGIHASHI *et al.,* 2001; OBROSOVA *et al.,* 2002), ativação inadequada da PKC (PRICE *et al.,* 2004) e PARP (LI *et al.,* 2005; OBROSOVA *et al.,* 2008), produção e acúmulo de AGEs (TANJI *et al*., 2000), estresse oxidativo/nitrosativo (CAMERON *et al.,* 2001; COPPEY *et al.,* 2001; OBROSOVA *et al.,* 2001) e disfunção mitocondrial (ZHANG *et al.,* 2012). Compostos carbonil reativos podem causar a depleção de GSH e inativação de enzimas antioxidantes, levando os neurônios a um estado pró oxidativo (KALAPOS *et al*., 2008).

O MG, por ser um produto do metabolismo intermediário, é sempre produzido mesmo em condições normais, sendo a sua principal fonte a fragmentação não-enzimática de triosefosfatos (PHILLIPS & THORNALLEY, 1993b). A sua exata concentração intracelular nos neurônios ainda não é

conhecida, mas o MG tem sido encontrado em fluidos corporais e tecido de pacientes e animais diabéticos em uma concentração de cerca de 400 μM (LAPOLLA *et al*., 2003a). Na linhagem de células CHO, derivadas de ovário de hamster chinês, concentrações por volta de 300 μM foram mensuradas, a maior parte ligado a resíduos de cisteína e GSH (GARCÍA DE ARRIBA *et al*., 2007). O GA é formado pela degradação oxidativa da glicose, a partir de proteínas glicadas, peroxidação lipídica, auto-oxidação de aminoácidos e pelos neutrófilos durante a fagocitose (AL-ENEZI *et al*., 2006; TOBLER & KOEFFLER, 1991). O GA sofre enolização e auto-oxidação, dando origem ao GO com concomitante produção de radical superóxido (ROBERTSON *et al*., 1981; SAKURAI & TSUCHIYA, 1988).

Compostos dicarbonil como MG, GA e GO têm sido citados como possíveis mediadores de danos oxidativos na progressão da ND, além terem sido associados a doenças neurodegenerativas e ao processo de envelhecimento (GARCÍA DE ARRIBA *et al*., 2006). A citotoxicidade dessas glicotoxinas já foi descrita em fibroblastos, macrófagos, células beta pancreáticas, neurônios e outras células de mamíferos (AMICARELLI *et al*, 2003; AL-ENEZI *et al*, 2005; WANG *et al*., 2009; YANG *et al*., 2011; KOIZUMI *et al*., 2011). Sabe-se que o MG inibe várias enzimas chave do metabolismo, como ATPases (MIRA *et al*., 1991; DERHAM *et al*., 2003), gliceraldeído-3-fosfato-desidrogenase (HALDER *et al*. 1993), complexo I da cadeia respiratória mitocondrial (BISWAS *et al*., 1997; ROSCA *et al*., 2002), GR e LDH (MORGAN *et al*., 2002). Estudos recentes mostraram que as células de neuroblastoma SH-SY5Y são bastante sensíveis ao MG devido à ineficiência de sua habilidade antioxidante e detoxificante (LEE *et al*., 2012; LI *et al*., 2011; GARCÍA DE

ARRIBA *et al*., 2007; KUHLA *et al*., 2006). Ainda não existem estudos investigando a toxicidade do GA às células SH-SY5Y, porém demonstrou-se em cultura primária de neurônios que essa glicotoxina afeta a sobrevivência neuronal, a produção de neuritos e a morfologia celular (LUO *et al*., 2002).

O tratamento da linhagem de neuroblastoma humano SH-SY5Y com AR durante 7 dias induz a diferenciação dessas células, as quais passam a apresentar morfologia neuronal e a expressar marcadores como TH, NSE e NeuN (LOPES *et al*., 2010). No entanto, apesar das células SH-SY5Y diferenciadas parecerem ser um modelo mais apropriado para estudos de mecanismos moleculares envolvidos em processos neurodegenerativos, a toxicidade de compostos carbonil reativos, como MG e GA, nunca foi testada neste modelo experimental. Assim sendo, o objetivo deste estudo foi investigar os mecanismos oxidativos envolvidos na toxicidade do MG e GA em células SH-SY5Y diferenciadas. Nossos dados serão discutidos em comparação com resultados obtidos através do tratamento da linhagem diferenciada de neuroblastoma humano SH-SY5Y com a neurotoxina 6-hidroxidopamina (6-OHDA) (*vide anexo*). A 6-OHDA é uma toxina muito bem estabelecida como oxidante de células neuronais (BOVÉ *et al.,* 2005) e, assim sendo, é possível que a sequência de eventos moleculares que leva a morte celular pela ação da 6-OHDA e pelas glicotoxinas seja similar.

Nossos resultados demonstraram que o tratamento com compostos carbonil reativos têm efeitos citotóxicos nas células SH-SY5Y diferenciadas, induzindo estresse oxidativo e morte celular por apoptose. As citotoxicidades do MG e GA mostraram-se dose dependente. Sabe-se que em neurônios altas concentrações de MG e grandes períodos de incubação são necessários para

induzir citotoxicidade (SHANGARI & O'BRIEN, 2004; SHINPO *et al*., 2000; KIKUCHI *et al*., 1999); esse dado da literatura está de acordo com o nosso resultado de que uma dose de 1350 μM de MG é necessária para induzir a perda de viabilidade de 50% das células em um tratamento de 24 horas. Por outro lado, o GA parece ser bem mais tóxico, possuindo um valor de DL_{50}= 115μM, provavelmente, devido ao fato dessa glicotoxina ser altamente reativa. O GA reage tão rapidamente com resíduos proteicos que a quantificação de sua forma livre é praticamente impossível (GLOMB & MONNIER, 1995). No entanto, ao compararem-se os dados de citotoxicidade das glicotoxinas com os da a 6-OHDA, em que o valor de DL_{50} é de apenas 15 μM, verifica-se que esta última possui um potencial de dano muito maior (ver Figura 1 do anexo1).

Já está bem estabelecido que os efeitos prejudiciais do MG em neurônios são devidos a indução da produção de ER, o que leva as células a uma morte por estresse oxidativo (DI LORETO *et al*., 2004; KOIZUMI *et al*., 2011). Estudos sobre a capacidade antioxidante demonstraram que a suscetibilidade dos neurônios ao MG está relacionada a sua ação debilitante sobre os sistemas antioxidantes, causando a diminuição da atividade de enzimas como da catalase, superóxido-dismutase (SOD) e GPx e, também, a depleção do GSH intracelular (DI LORETO *et al*., 2004; 2008). Relatos da toxicidade do GA, também, têm relacionado o envolvimento do estresse oxidativo, sendo descritos fenômenos como o aumento do radical superóxido, peroxidação lipídica, e acúmulo de proteínas carboniladas (AL-ENEZI *et al*., 2006; LORENZI *et al*., 2010a; LORENZI *et al*., 2010b). A 6-OHDA, uma vez dentro da célula, auto-oxida-se gerando inúmeras ER e induzindo massivo estresse oxidativo (GOMEZ-LAZARO *et al*., 2008).

Neste estudo, as citotoxicidades do MG e GA foram precedidas da diminuição dos níveis de GSH e aumento da produção de ER, sendo que os dois eventos ocorreram nos primeiros 90 minutos de exposição a essas glicotoxinas. Este rápido aumento na produção de ER sugere que o estresse oxidativo possa ser um dos principais culpados pela toxicidade do MG e GA; e, essas ER podem estar atuando como sinalizadoras da morte celular como já foi sugerido em estudos feitos com outros tipos celulares (DU *et al*., 2001; OKADO *et al*., 1996). A ocorrência deste rápido desequilíbrio redox tem sido relatada por vários pesquisadores (AMICARELLI *et al*., 2003; GARCIA DE ARRIBA *et al*., 2006; DI LORETO *et al*., 2008; OKOUCHI *et al*., 2009). Isto não ocorre quando as células são tratadas com a 6-OHDA. Apesar de saber-se ser essa neurotoxina um oxidante muito mais potente, o aumento da produção de ER leva cerca de uma hora e meia para começar; entretanto, uma vez iniciado verifica-se que ele é bastante intenso, gerando valores de fluorescência bem maiores do que os obtidos com o tratamento com as glicotoxinas (ver Figura 2 do anexo). Este atraso no início da detecção do aumento do DCF deve-se, provavelmente, a necessidade da 6-OHDA ser primeiramente transportada para dentro das células pelos DAT.

Passadas seis horas do tratamento, os níveis de GSH estão voltando aos valores normais, entretanto, a detecção de ER pelo DCF continua aumentada, mesmo passadas 24 horas do tratamento. Provavelmente isto ocorra como uma consequência da glicação proteica e lipídica causada pelo MG e GA, que produz AGEs e leva ao estresse oxidativo. O MG induziu uma maior e mais duradoura diminuição nos níveis de GSH do que o GA. Este resultado pode ser explicado através na análise do tipo de sistema de

detoxificação envolvido com cada glicotoxina. O MG é enzimaticamente metabolizado pelo sistema da glioxalase, o qual é composto por duas enzimas: a glioxalase I e a glioxalase II (THORNALLEY, 1996; THORNALLEY, 2003). Este sistema utiliza GSH como um substrato para converter MG a D-lactato. Assim, altas doses de MG podem gerar uma hiperatividade deste sistema causando a depleção de GSH. Todavia, o mecanismo de detoxificação do GA ainda permanece desconhecido. Um estudo recente mostrou que a álcool desidrogenase, enzima capaz de reduzir pequenas moléculas de aldeídos como o acetaldeído e formaldeído, pode converter o GA em etileno glicol usando NADH como cofator (JAYAKODY *et al*., 2012).

A diminuição nos níveis de GSH e o aumento de ER gerados pelos tratamentos com MG e GA sugerem que há o envolvimento do estresse oxidativo na citotoxicidade dessas glicotoxinas e, que isto é um evento inicial do processo de morte. Para investigar melhor essa relação, foi avaliada a ativação das caspases-3 -8 e -9; sendo que todas elas já foram relacionadas aos mecanismos de morte celular do MG ou GA (AMICARELLI *et al*., 2003; OKOUCHI *et al*., 2009; MOHEIMANI *et al*., 2010). A marcação com os compostos fluorescentes Hoechst 33342 e iodeto de propídeo mostraram que a apoptose é o principal tipo de morte celular gerada por essas glicotoxinas, resultado esse que foi complementado pela intensa ativação de caspases encontrada. As células SH-SY5Y diferenciadas ao serem tratadas com MG ou GA sofrem morte celular programada através de ambas as vias: a intrínseca e a extrínseca, como foi demonstrado pela ativação da caspase-9 e -8, respectivamente. A maior parte dos dados existentes na literatura relata que a apoptose mediada pelo MG ocorre pela via intrínseca, a qual é tipicamente

ativada pelo estresse oxidativo (AMICARELLI *et al*., 2003; OKOUCHI *et al*., 2009). Mesmo assim, a ativação da via extrínseca da apoptose pelo MG já foi relatada (DI LORETO, 2008). Em células SH-SY5Y parece que MG e GA induzem apoptose por ambas as vias, a mitocondrial e a citosólica, o que não é verificado no tratamento com a 6-OHDA, a qual ativa apenas a via intrínseca de apoptose, o que é bastante característico de moléculas que induzem grande estresse oxidativo. Além disso, podemos notar que o tratamento com MG ativa caspase-3 a partir dos 90 minutos de exposição. Essa rápida ativação da apoptose pode explicar porque encontramos células com o núcleo condensado que sofreram a incorporação de iodeto de propídeo; e, explica também, a ocorrência de um aumento na liberação de LDH. Lembrando que a liberação de LDH foi quantificada apenas após 24 horas de tratamento, se a apoptose ocorreu muito rapidamente, no momento da detecção os corpos apoptóticos poderiam estar deteriorados – o que podemos chamar de apoptose tardia (POON *et al*., 2010). No tratamento com GA não foi detectado aumento significativo da ativação da caspase-3, provavelmente, isto deve-se ao fato da análise ter sido feita apenas nas primeiras 6 horas de exposição, podendo essa caspase estar sendo ativada posteriormente.

A literatura diverge bastante quanto ao tempo de perda do potencial da membrana mitocondrial (MMP) gerado pelo MG e GA. OKOUCHI (2009) mostrou que a liberação do citocromo *c* ocorre nos primeiros 30 minutos após o tratamento com MG, enquanto GARCIA DE ARRIBA (2006) verificou a perda do potencial da membrana mitocondrial 24 horas depois da exposição ao MG. Possivelmente essas diferenças são devidas ao tipo celular estudado e suas respectivas capacidades antioxidantes. As células tratadas com 6-OHDA

apresentam a perda do MMP após 6 horas de tratamento, dado que coincide com o tempo em que se verifica a ativação da caspase-3 (ver Figura 4 do anexo). Este dado reforça que a toxidade da 6-OHDA é extremamente via estresse oxidativo, afetando principalmente a mitocôndria.

Analisando resultados de outros estudos podemos observar que diferentes tipos celulares têm diferentes sensibilidades ao MG e GA, o que pode ser atribuído a uma variabilidade tipo-celular específica na capacidade de combater os efeitos adversos das ER. Para ir mais fundo neste quesito, avaliamos a contribuição de diferentes sistemas antioxidantes no metabolismo do MG e GA, isto foi feito através da inibição das enzimas TrxR e GR. Foram escolhidas estas enzimas, porque as mesmas fazem parte dos sistemas tiorredoxina (Trx) e glutarredoxina (Grx), respectivamente; e, estes sistemas são os principais envolvidos na proteção contra danos oxidativos a sulfidrilas (BERNDT *et al*., 2007), como pode ser visto na figura 4, sendo que está bem descrito que o MG tem alta reatividade com este grupamento (DI LORETTO *et al.*, 2008).

A inibição dessas enzimas foi seguida de um tratamento com doses subletais do MG e GA. Através dos resultados obtidos podemos concluir que a TrxR tem um papel de maior importância na defesa das células SH-SY5Y diferenciadas contra a citoxicidade do MG do que contra o GA. Isto pode ser inferido, porque a inibição dessa enzima aumentou em 31% a morte celular frente ao desafio com MG, enquanto o aumento da toxicidade do GA foi de apenas 15%. Entretanto, a GR parece ter a mesma importância na defesa celular contra MG e GA, já que sua inibição potencializou a perda de viabilidade celular em 25% para ambas glicotoxinas. A inibição de ambas as

enzimas aumentou a citotoxicidade da 6-OHDA; provavelmente, pelo fato dessa neurotoxina induzir um estresse oxidativo muito intenso qualquer alteração na capacidade antioxidante é extremamente danosa à célula.

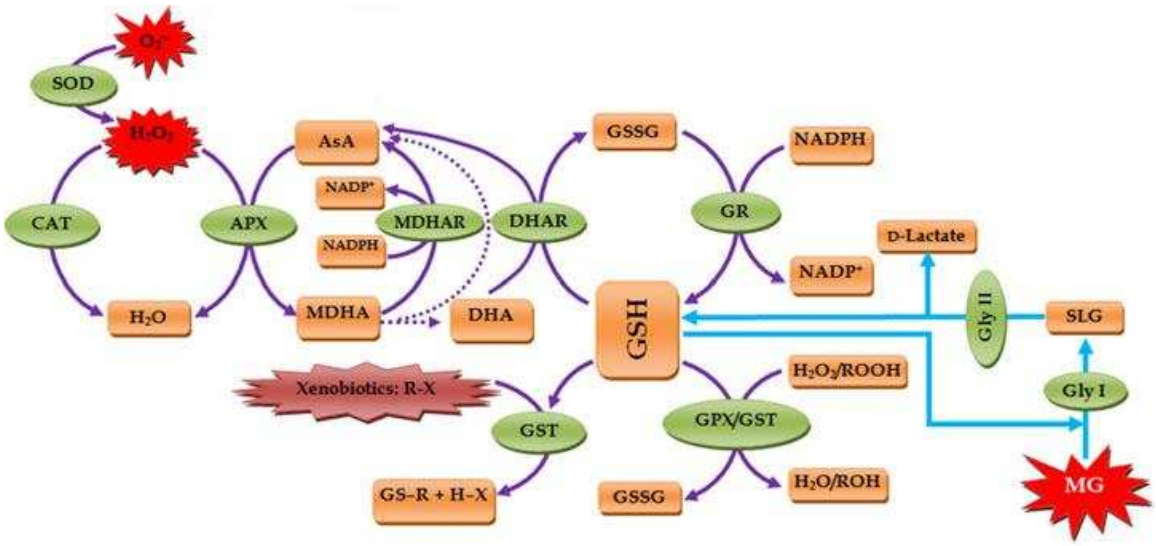


Fig. 4: Ação coordenada de sistemas antioxidantes na defesa frente o tratamento com MG, tendo como molécula central a GSH (figura adaptada de HASANUZZAMAN *et al*, 2012). O_2^-: radical superóxido, SOD: superóxido dismutase, H2O2: peróxido de hidrogênio, CAT: catalase, APx: peroxidase, AsA: ascorbato, MDHAR: monodeidroascorbato-redutase, MDHA: monodeidroascorbato, DHA: deidroascorbato, DHAR: deidroascorbato-redutase, GSH: glutationa reduzida, GSSH: glutationa oxidada, GR: glutationa-redutase, GST: glutationa-S-transferase, GPX: glutationa-peroxidase, MG: metilglioxal, Gly I: glioxalase I, Gly II: glioxalase II, SLG: S-D-Lactoilglutationa.

Nossos resultados trazem novos dados sobre as vias envolvidas na toxicidade do MG e do GA, sendo essas vias potenciais alvos terapêuticos para a ND.

5. CONCLUSÃO

Nossos resultados mostraram que o tratamento das células diferenciadas SH-SY5Y com MG ou GA causou uma rápida diminuição do conteúdo de GSH, um aumento na produção de ER, que levou a uma rápida ativação de caspase- 8 e -9 e, posterior ativação da caspase-3. Conjuntamente, estes dados sustentam a hipótese de que o MG e o GA provocam a formação de um ambiente pró-oxidante nas células. Entretanto, o estresse oxidativo induzido por essas glicotoxinas é muito menos intenso do que o produzido pela neurotoxina 6-OHDA. Ainda, as vias de toxicidade e morte celular ativadas por MG e GA não são as mesmas. Podemos dizer que o MG e o GA possuem diferentes formas de detoxificação, sendo este estudo um novo *insight* sobre os mecanismos moleculares envolvidos na neurotoxicidade dessas glicotoxinas.

6. PERSPECTIVAS

Como perspectivas deste trabalho temos:

- a verificação da atividade das caspases, principalmente a caspase-3, em tempos de tratamento com o MG e o GA superiores a seis horas;

- a realização do ensaio de JC-1 durante as primeira 24 horas de tratamento com MG e GA, para avaliar melhor a participação da disfunção mitocondrial na citotoxicidade dessas glicotoxinas;

- avaliação da atividade das glioxalases I e II após o tratamento com MG e GA, para verificar a importância de sua participação na detoxificação dessas glicotoxinas.

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8. ANEXOS

8.1 ANEXO 1

O artigo científico elaborado conforme as normas da revista The Journal of Biological Chemistry.

``OXIDATIVE STRESS AND PEROXIDASE SYSTEMS IN 6-HYDROXYDOPAMINE NEUROTOXICITY IN DIFFERENTIATED HUMAN SH-SY5Y NEUROBLASTOMA CELLS: INSIGHTS INTO THE PATOPHYSIOLOGY OF PARKINSON DISEASE ``

OXIDATIVE STRESS AND PEROXIDASE SYSTEMS IN 6-HYDROXYDOPAMINE NEUROTOXICITY IN DIFFERENTIATED HUMAN SH-SY5Y NEUROBLASTOMA CELLS: INSIGHTS INTO THE PATOPHYSIOLOGY OF PARKINSON DISEASE

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Oxidative stress is related to many neurodegenerative processes as Parkinson disease. It can activate signaling pathways and modulate a variety of cellular activities. These effects are believed to involve, at a molecular level, the reversible post-translational modification of critical protein thiols. However many of the initial targets of oxidation remain unidentified. In an effort to discover novel oxidant sensitive proteins we adapted a technique to fluorescently label oxidized thiol proteins then separate them by two-dimensional electrophoresis (2-DE). Cells were disrupted in the presence of N-ethylmaleimide (NEM) to block the reduced thiol proteins and tris(2-carboxyethyl)phosphine (TCEP) was added to reduce the oxidized thiol proteins before labeling with 5-iodoacetamidofluorescein (5-IAF). 2-DE was used to resolve the labeled samples. We applied the method to differentiated SH-SY5Y human neuroblastoma cells and examined the effect of 6-hydroxydopamine (6-OHDA) treatment on the oxidized and reduced thiol protein profiles. A small percentage of protein thiols were already oxidized in untreated cells. Exposure of cells to 15 µM 6-OHDA (LD_{50} value of this drug) for 6 h led to a dramatic increase in reactive species formation, caspase-3 and -9 activation, mitochondrial depolarization and thiol protein oxidation. The antioxidant enzymes glutathione reductase (GR) and tioredoxin reductase (TrxR) have the same importance in 6-OHDA detoxification. These results are useful in gaining insight into pathophysiological mechanisms of neurodegeneration mediated by 6-OHDA.

Oxidative stress is defined as the imbalance between biochemical processes leading to the production of reactive oxygen species (ROS)[1] and those responsible for the removal of ROS, the so-called antioxidant cascade[1]. The central nervous system is particularly vulnerable to oxidative insult on account of the high rate of O_2 utilization, the relatively poor concentrations of classical antioxidants and related enzymes, and the high content of polyunsaturated lipids[2]. In addition, there are regionally high concentrations of redox-active transition metals capable of the catalytic generation of ROS[3]. Thus, it is not surprising that oxidative stress is a common discussion point for neurodegenerative disease, where damage to neurons can

[1] List of Abbreviations used: ROS, reactive oxygen species; AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotropic lateral sclerosis; DA, dopamine; 6-OHDA, 6-hydroxydopamine; JC-1, 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolcarbocyanine iodide; FBS, fetal bovine serum; RA, retinoic acid; DCF-DA, 2,7-dichlorofluorescein diacetate; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; 5-IAF, 5-iodoacetamidofluorescein; NEM, N-ethilmaleimide; TCEP, tris(2-carboxyethyl)phosphine; LD_{50}, letal dose to 50% of the cells; 2-DE, two dimension electroforesis; IEF, isoeletric-focusing; DMSO, dimethilsulphoxide; RS, reactive species.

reflect both an increase in oxidative processes and a decrease in antioxidant defenses[4].

Progressive degeneration of a subset of neurons is the pathologic hallmark of adult-onset neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS)[5]. Growing evidence points to the involvement of reactive species in mediating neuronal death in these diseases[6]. Elucidation of the intracellular pathways involved in the formation of oxidants in neurons may be important not only for understanding the pathophysiologic basis for neuronal death in these disorders, but also for devising rational pharmacologic strategies to slow or prevent neuronal degeneration.

Parkinson's disease (PD) is an age-related neurodegenerative disease, characterized by relatively selective nigrostriatal dopaminergic degeneration[7]. The clinical symptoms of PD are resting tremor, rigidity, bradykinesia and postural instability. The pathological characteristics are loss of dopamine (DA) neurons and the presence in the *substantia nigra* of intracytoplasmic inclusions, known as Lewy bodies, which are consistently immunostained with antibodies to α-synuclein and ubiquitin[8].

6-OHDA, an oxidative metabolite of dopamine, is a neurotoxin, which has been broadly used to generate experimental models of Parkinson's disease[9-11]. Although there is a consensus in the ability of 6-OHDA to induce cytotoxicity in different cell types, the mechanism involved is still controversial[12,13]. Among the mechanisms discussed, the generation of ROS is the most accepted[10].

Thiol groups are critical parts of proteins involved in cell signaling pathways, their oxidation alters the activity or binding properties of proteins. Although some proteins have been identified as redox-sensitive, it is not clear how many proteins become oxidized in a cell during signaling, or which changes are critical to the subsequent response[20].

In this study we validated a sensitive technique for identifying thiol proteins that become oxidized in cells during normal metabolism or after being challenged with 6-OHDA. For these purposes we used the differentiated human neuroblastoma cell line SH-SY5Y as experimental model. We previously demonstrated that these cells present all desired neuronal markers (*e.g.* extensive neurites outgrown and the biochemical markers tyrosine hydroxilase - TH, neuron nuclear protein - NeuN, neuron specific enolase - NSE) with the concomitant absence of undifferentiated cellular markers (*e.g.* nestin immunocontent), being considered a more suitable experimental model for studying the molecular and cellular processes that commit dopaminergic neuronal cell, like PD[21].

EXPERIMENTAL PROCEDURES

Chemicals – JC-1 and SilverQuest™ Staining Kit were from Invitrogen (Carlsbad, CA). TCEP was from Pierce (Rockford, IL). Complete protease inhibitor cocktail tablets was from Roche® (Mannheim, Germany). Micro Bio-Spin 6 chromatography columns were from BioRad. 7 cm IPG Strips pH 3–10 were from GE Healthcare Bioscience (Uppasala, Sweden). IPG buffer, pH 3-10NL was from Amersham Biosciences (Uppasala, Sweden). DCF, 5-iodoacetamidofluorescein, iodoacetamide, DTT, NEM, MTT, ultrapure urea, thiourea and the other reagents were from Sigma (Saint Louis, MO).

Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP Brazil).

Cell culture and differentiation – Exponential growing human neuroblastoma cell line SH-SY5Y, obtained from ATCC (Manassas, VA, USA), were maintained in a mixture 1:1 of Ham's F12 and Dulbeco Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 0.28 μg/μL gentamicin and 250 μg amphotericin B, in a humidified atmosphere of 5% of CO_2 in air at 37ºC. Cell medium were replaced every three days and cell were sub-cultured once they reached 80% confluence. After 24 hours of cell plating, differentiation was induced by lowering the FBS in culture medium to 1% plus the

addition of 10 μM retinoic acid (RA) during 7 days. This treatment was replaced each 3 days to replenish RA in culture media. All treatments were performed when cells were ~75% confluence. For the MTT and DCF assay the cells were plated in a 96-well plated at density of 2 x 10^4 cells per well. For 2D-electrophoresis, the cells were seeded into bottles of 75 cm^2 at density of 5 x 10^6 cells.

6-OHDA cytotoxicity – 6-OHDA was freshly prepared in 0.1% ascorbic acid to avoid oxidation. Cytotoxicity of 6-OHDA was evaluated by exposing cells to different concentrations of this neurotoxin for 24 hours at 37ºC, and cell viability was estimated by the quantification of the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a blue formazan product by cellular dehydrogenases[22]. At the end of the treatment, the medium was discarded and the cells were washed three times with phosphate buffer (PBS). A new medium containing 0.5 mg/mL MTT was added. These cells were incubated for 1 hour at 37ºC. This medium was discarded and DMSO was added to solubilize the formazan crystals for 30 minutes. Absorbance was determined at 560 nm and 630 nm in a SoftMax Pro Microplate Reader (Molecular Devices®, USA).

Total reactive species – 2,7-dichlorofluorescein diacetate (DCF-DA) 1,1 mM solution was made freshly in PBS from a stock solution (30 mM) made in DMSO. The solution was added to the cells in medium to a final concentration of 100 μM, and the plates were incubated for 1 hour in an atmosphere of 5% CO_2 at 37ºC to load cells with DCF. Then, the medium was discarded and a new medium was added with 6-OHDA LD_{50} value (15 μM) and cotreated with 5 mM of glutathione (GSH) or not. DCF was excited at 485 nm, and fluorescence emission at 538 nm was recorded using a SoftMax Pro fluorescence plate reader (Molecular Devices®, USA).

Mitochondrial Membrane Potential ($\Delta\Psi_m$) – Differentiated SH-SY5Y cells were treated by different times with the LD_{50} value of 6-OHDA (15 μM) to evaluate the mitochondrial depolarization, as previously described[23]. Cells (0.5 x 10^6 cells/mL) were incubated for 20 minutes at 37ºC with 10 μg/μL a lipophilic cationic probe 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), centrifuged, washed once with PBS, transferred to a 96-well plate (10^5 cells/well), and assayed using SoftMax Pro fluorescence plate reader (Molecular Devices®, USA) with the following settings: excitation at 485 nm, emission at 540 and 590 nm, and cut-off at 530 nm. $\Delta\Psi_m$ was estimated using the ratio of 590 nm (J-aggregates)/540 nm (monomeric form).

Inhibition Studies- Differentiated SH-SY5Y cells were pretreated for 30 minutes with aminotriazole (AT), acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylthiocarbonylamino)phenylthiocarbamoylsulfanyl]propionic acid hydrate S,S′-[1,4-Phenylenebis(iminocarbonothioyl)]bis[N-acetyl-L-Cysteine] hydrate) (2-AAPA), or auranofin (AU) - inhibitors of catalase, GR and thioredoxin reductase TrxR, respectively - and the specific enzymatic activity and cell viability was measured. A non lethal dose of each drug, able to inhibit their respective enzymes activities, was established (data not shown). The contribution of each enzymatic system in 6-OHDA toxicity was evaluated, the differentiated cells were pretreated for 30 min with the inhibitors (10 μM of 2-AAPA or 0,5 μM of AU) and exposed to a sublethal dose of 6-OHDA (μM) for 24 hours. Cell viability was determinated by MTT assay.

Fluorescence labeling of thiol proteins – Cells were scraped out from de culture flask, collected by centrifugation at 1000*g* for 5 min and the supernatant was discarded. The pellet was washed with PBS and centrifugated again at 1000*g* for 5 min. The supernatant was discarded and the cells were resuspended in extract buffer (10 mM Tris, protease inhibitors, pH 7.4). The extract was sonicated and the protein content was measured. Reduced protein thiols were blocked by incubating the extract with 100 mM N- ethylmaleimide (NEM) solution for 20 minutes. After, NEM excess was removed by three times iced acetone precipitation. The pellet was resuspended in extract buffer. Oxidized thiols (disulphides or sulphenic acid

groups) were reduced by adding Tris(2-carboxyethyl) phosphine (TCEP) to a final concentration of 1 mM and incubated for 15 minutes in ice. A stock solution of 10 mM 5-iodoacetamidofluorescein (5-IAF) was made up in DMSO and added to the extract to a final concentration of 200 µM. The extract was incubated in the dark on ice for 15 min. Subsequent steps were carried out with minimal exposure of the samples to light. To remove excess 5-IAF and salts another three times iced acetone precipitation was proceed and the pellet was resuspended in rehydration solution (7 M urea, 2 M thiourea, 10 mM DTT, 4% CHAPS w/v, 0.2% Biolytes 3–10 NL v/v and bromophenol blue) for 2-D Electrophoresis.

2-D Electrophoresis – Extracts containing 50 µg of protein in rehydration solution were absorbed overnight onto pH 3–10/NL Immobiline Drystrips. Isoelectric focusing (IEF) was carried out with 9083 Vh. For the second dimension, focused IPG strips were equilibrated for 15 min in equilibration solution (6 M urea, 36% glycerol v/v, 2% SDS w/v, 0.002% bromophenol blue and 50 mM Tris-HCl, pH 6.8) containing 2% DTT w/v, then for 15 min in equilibration solution containing 2.5% iodoacetamide. The strips were placed on SDS-PAGE gels (10% separating gel and a 4% stacking gel) and a 0.5% agarose solution w/v was placed onto the strips. SDS-PAGE was carried out in the dark with a constant current of 20 mA per gel. After completion of electrophoresis, gels were scanned using a Galiance 600 PerkinElmer® imaging system. Gels were then fixed and stained with silver nitrate or Comassie Brilliant blue.

Protein quantification – The proteins contents were measured by Bradford assay[24].

RESULTS

Citoxicity of 6-OHDA in differentiated SH-SH5Y cells – Cytotoxicity was evaluated using the MTT assay. Cells were treated with different concentrations of 6-OHDA for 24 hours and 15 µM represent the LD_{50} drug value for this cell line, as previously established. 0.1% ascorbic acid was tested as vehicle control. A decreased survival was seen in cells treated with 6-OHDA when compared to control (Fig. 1).

Reactive species (RS) production – Time course experiment were performed using the DCF dye to evaluate the generation of reactive species by 6-OHDA (Fig. 2). The 6-OHDA treatment caused an increase in RS production. This increase started between 2 to 6 h (Fig. 2A) of treatment and followed for 24 h (Fig. 2B) and it was reduced by GSH cotreatment.

Mitochondrial membrane potential – Since mitochondria dysfunction is a landmark of many neurodegenerative diseases, including PD, we performed a time course experiment to determine the loss of mitochondrial membrane potential in differentiated SH-SY5Y cells challenged with 6-OHDA (Fig. 3A). In accordance to RS production data (Fig. 2A), mitochondrial depolarization occurred 6 hours after 6-OHDA treatment (Fig. 3A). Taken into account the time course data on RS generation and mitochondrial dysfunction, we decided to use protein extract isolated from cells treated by 6 h with 6-OHDA treatment to perform redox proteome analysis.

6-OHDA activate Caspase-9 and -3 -To test if apoptosis was responsible for the death of differentiated SH-SH5Y cells observed after 6-OHDA treatment and what pathway is involved, we investigated caspases-3, -8 and -9 activities in these cells using a synthetic substrate that contains the cleavage site recognized by these proteases. A time curve among 1,5 hours and 12 hours had been performed in order to determine the kinetics of caspase activation. 6-OHDA treatment has activated caspase-9 after 1,5 h and caspase-3 after 6 hours (Fig. 3 B). No difference was detected in the caspase-8 activity.

Inhibition of GR and TrxR improve 6-OHDA toxicity- To evaluate the contribution of different peroxidases systems in the defense of differentiated SH-SY5Y cells against 6-OHDA cytotoxicity we inhibited GR and TrxR activities, challenged the cells with sublethal doses of this neurotoxin and evaluated the cell viability after 24 hours. For this purpose, we first established the best conditions to

specifically inhibit each peroxidase systems and to determine the toxicities of these inhibitors (data not shown). The pretreatment with both enzymes inhibitors, AU and 2-AAPA, have increased 6-OHDA toxicity in 25%, not showing difference of importance in the detoxification this neurotoxin (Fig. 4).

Redox Proteome Validation – Firstly we performed a validation of the redox proteome protocol (Fig. 5), were we perform the blockage of reduced thiols and then the reduction of oxidized thiols (NEM/TCEP) (lane1), the total reduction and then the total blockage of thiols (TCEP /NEM) (lane 2) and we treated cells with 200 μM of H_2O_2 for 1 hour and then performed the blockage and reduction like lane 1 (H_2O_2 /NEM/TCEP) (lane 3). Then 20 μg of each treated protein extract was labeled with 5-IAF and used to perform SDS-PAGE.

A series of intense bands were observed in lane 1 (Fig. 5), indicative of the large number of reduced thiol proteins present in cells. In lane 2 (Fig. 5) we can notice that the majority of these reduced thiols were blocked comparing with lane 1. When cells extracts were treated with 200 μM of H_2O_2 more bands appeared when compared with control (compare lane 3 with lane 1, Fig. 5). Thus we demonstrate that our redox proteome protocol is suitable to analyze fluctuations in the redox status of proteins thiols.

2D-Electrophoresis – To visualize the different oxidation target proteins we performed the redox proteome. Differentiated cells were treated with the 6-OHDA LD_{50}, redox proteome protocol was performed and this extract was run in parallel with untreated samples (Fig. 6). When control and treated samples were separated in two dimensions by isoelectric focusing (IEF) and SDS-PAGE (Fig. 6, A and B), it was possible to detect many individual oxidized thiol proteins. Some proteins showed an increase in 5-IAF labeling after treatment of cells with 6-OHDA.

Total protein staining of the gels with Coomassie brilliant blue or silver nitrate revealed a markedly different protein pattern indicating the specific labeling of oxidized thiol proteins by 5-IAF (data not shown). Protein staining showed that protein loading was comparable for treated and untreated cells (not shown) so would not account for the increase in observed 5-IAF labeling.

DISCUSSION

Thiol proteins are important in cellular antioxidant defenses and redox signaling. Reactive oxidants cause selective thiol oxidation and can modulate cellular function, but relative sensitivities of different cell proteins and critical targets are not well characterized. The technique to fluorescently label oxidized thiol proteins and separation by 2-DE is a useful tool to discover novel oxidant sensitive proteins for further validations experiments to get into the mechanisms of redox signaling.

Using a fluorescence labeling procedure, we have detected changes in the pattern of reversibly oxidized thiol proteins in differentiated human neuroblastoma SH-SY5Y cells after treatment with 6-OHDA. The viability assay showed a comparable decrease in cell survival in 6-OHDA treatment. Moreover it induced an increase in RS production that is involved with the decrease in cell viability.

Time course experiments showed that mitochondrial depolarization occurs 6 hours after 6-OHDA treatments. Interestingly, in DCF experiments we can note that the amount of reactive species (RS) has a great increase at this time. As previously described, the resultant oxidative stress can potentially triggers the activation of p38 MAPK cascade, leading to lost of $\Delta\Psi m$ and cytochrome c release which results in caspase 9 and 3-dependent apoptosis. Also, we can see that caspase-9 was activated after 1,5 h of 6-OHDA exposition and caspase-3 after 6 hours. Caspase-8 was not activated by 6-OHDA treatment. Caspase-9 activation indicates a mitochondrial induced death, what is usual for toxins that cause massive oxidative stress. Caspase-3 activation happens at the same time of mitochondrial despolarization. Moreover, using our redox proteome protocol we were able to detect various oxidation targets in 6-OHDA treatment.

We have not attempted to characterize oxidant sensitive proteins in this study. However, once susceptible spots are identified, they can be subjected to standard proteomic analysis to give their isoeletrict point (pI) and molecular mass (*Mr*) and be characterized using techniques such as sequencing or in-gel proteolytic digestion followed by MALDI-TOF MS of extracted peptides.

In conclusion, 2-DE analysis of IAF labeled oxidized thiol proteins is useful in identifying novel targets of oxidation as well as to map patterns of changes in protein oxidation with different types of treatments.

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FIGURES LEGENDS

FIG. 1: **6-OHDA cytotoxicity in differentiated human neuroblastoma SH-SY5Y cell line**. Cells were exposed for 24 hours at 37ºC, and the cytotoxicity was evaluated using the MTT assay, as described in experimental procedures section. The data represent means ± SD for three experiments carried out in quadruplicates.

FIG. 2: **Time course measurements of reactive species generation in differentiated human neuroblastoma SH-SY5Y cell line by 6-OHDA treatment.** Cells were challenged with LD_{50} value of 6-OHDA (15 μM) and cotreated with 5mM of GSH. DCF assay was performed as described in experimental procedures section. Fluorescence was measured (A) during the first 6 hours and (B) after 24 hours of treatments. The data represent means ± SD for three experiments carried out in quadruplicates.

FIG. 3: **Mitochondrial membrane potential (ΔΨm) and caspases activation in differentiated human neuroblastoma SH-SY5Y cell line challenged by 6-OHDA treatment.** *(*A) Loss of membrane potential in differentiated cells of the neuroblastoma cell line SH-SY5Y challenged with 6-OHDA. Cells were treated with respective 6-OHDA LD_{50} value, harvested and incubated with JC-1 (10 μg/mL) at 37 ºC for 15 min. (B) Caspases-3, -8 and -9 activation was assayed as described in experimental procedures section. Measurements were performed after 1,5, 3h, 6h, 9h and 12h of 6-OHDA LD_{50} value exposition. The data represent means ± SD for three experiments carried out in quadruplicate.

FIG. 4: **Antioxidants systems contribution in defense against 6-OHDA toxicity in differentiated human neuroblastoma cell line SH-SY5Y.** Cells were pretreated for 30 minutes with (A) Au or (B) AAPA, the TrxR and GR inhibitors, respectively. Then, cells were exposed to a sublethal dose of 6-OHDA for 24 hours, and cell viability was assayed. Results were calculated as a percentage of values obtained for control cells. The data represent mean±SD for three experiments carried out in quadruplicates.

FIG. 5: **Optimization of experimental conditions for NEM blocking and IAF labeling of oxidized protein thiols, using SDS-PAGE**. Lane 1: Reduced thiols were first blocked with NEM and then the oxidized thiols were reduced with TCEP. Lane 2: Thiols were totally reduced with TCEP and then the total blockage was done with NEM. Lane 3: Cells were previously treated with 200 μM H_2O_2 for 1 hour and then the blockage and reduction like lane 1 was performed. 20 μg of each treated protein extract was labeled with 5-IAF and used to perform SDS-PAGE.

FIG. 6: **2-D PAGE separation of IAF labeled oxidized proteins in differentiated human neuroblastoma SH-SY5Y cell line without (A) and with (B) 6-OHDA treatment.** Differentiated cells were treated with the 6-OHDA LD_{50}, and redox proteome protocol was executed and this extract was run in parallel with an untreated or 6-OHDA-treated extract. Data present a representative experiment carried out in triplicates.

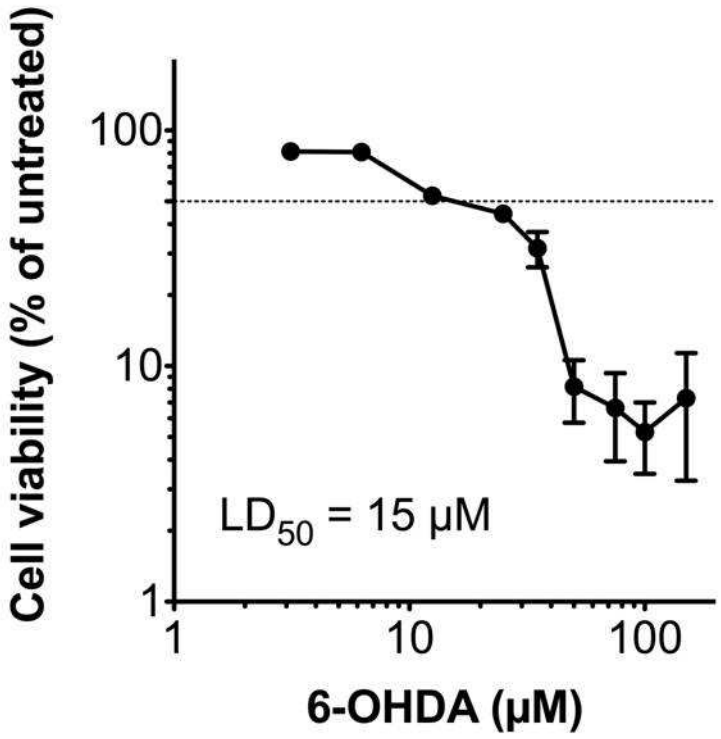

Cell viability (% of untreated)
100
10
1
LD50 = 15 µM
1
10
100
6-OHDA (µM)

Figure 1

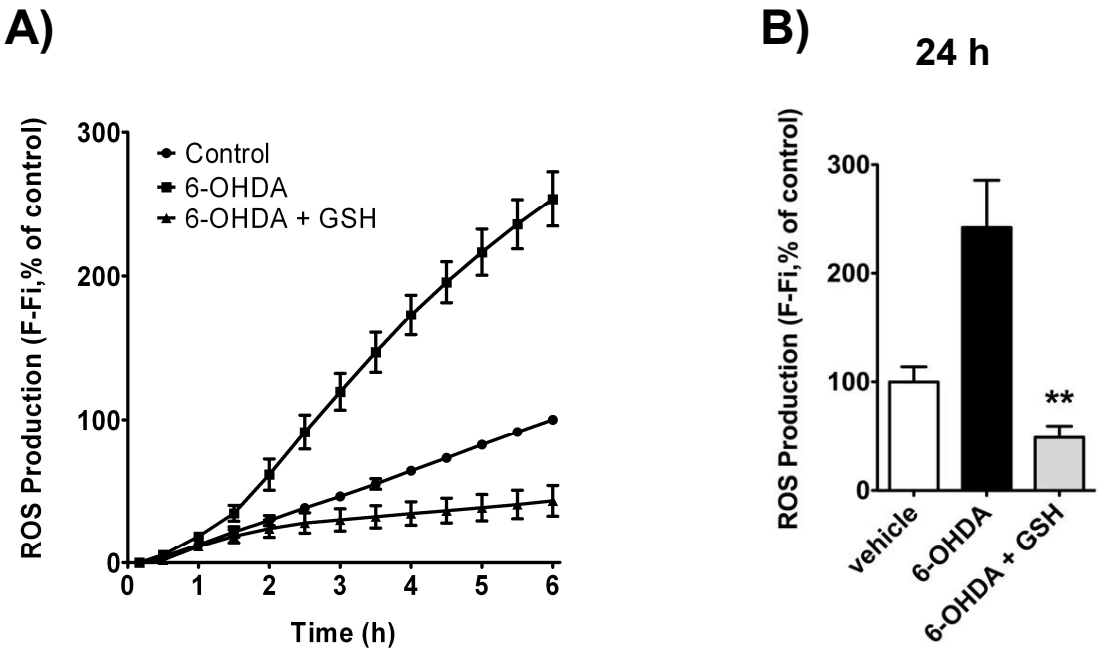
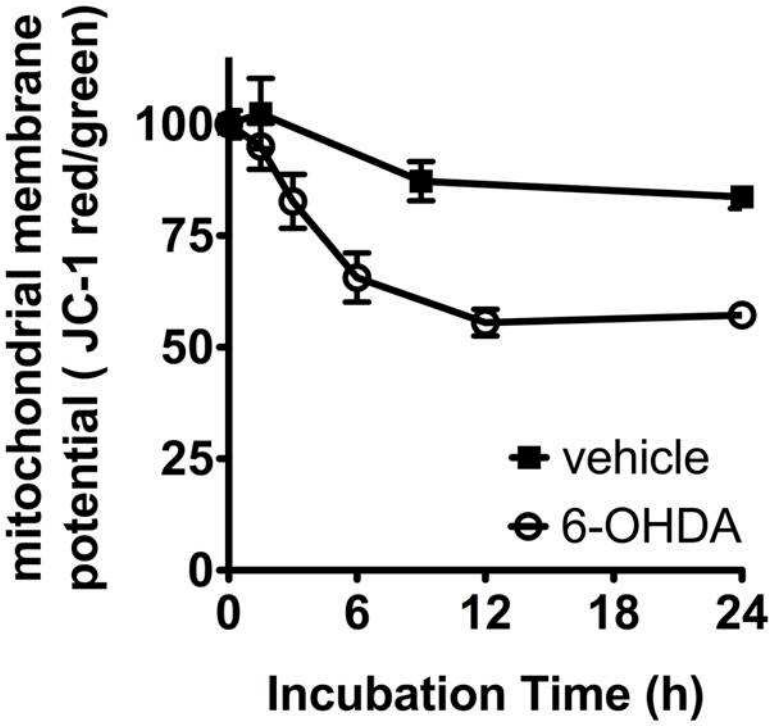

A)
B)
24 h
ROS Production (F-Fi,% of control)
300
200
100
0
Control
6-OHDA
6-OHDA + GSH
0
1
2
3
4
5
6
Time (h)
**
vehicle
6-OHDA
6-OHDA + GSH

Figure 2

A)


mitochondrial membrane potential (JC-1 red/green)
100
75
50
25
0
0
6
12
18
24
Incubation Time (h)
vehicle
6-OHDA

B)

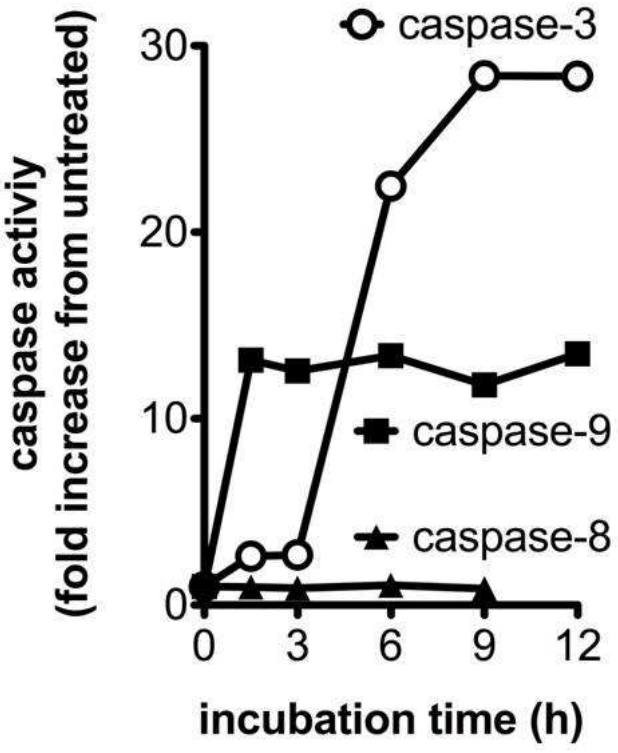
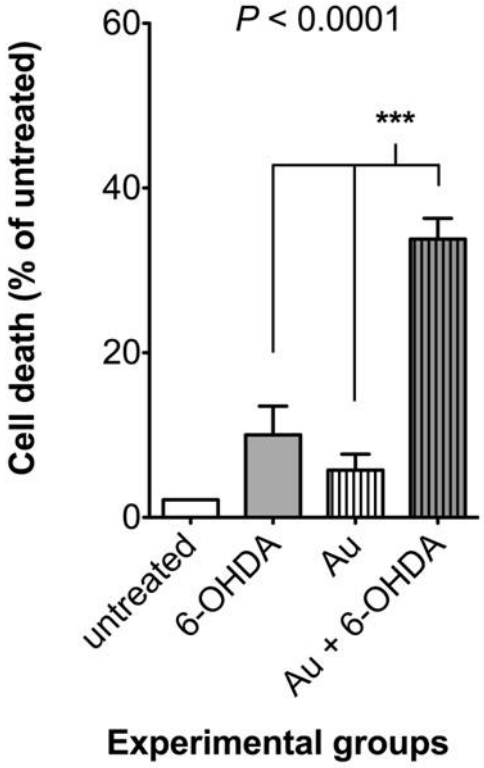

caspase activiy (fold increase from untreated)
30
20
10
0
0
3
6
9
12
incubation time (h)
caspase-3
caspase-9
caspase-8

Figure 3

A)


P < 0.0001
***
Cell death (% of untreated)
60
40
20
0
untreated
6-OHDA
Au
Au + 6-OHDA
Experimental groups

B)

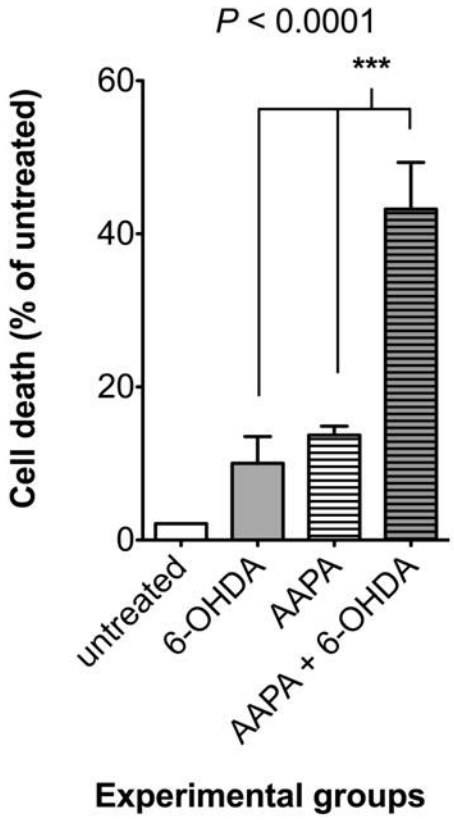

P < 0.0001
***
Cell death (% of untreated)
60
40
20
0
untreated
6-OHDA
AAPA
AAPA + 6-OHDA
Experimental groups

Figure 4

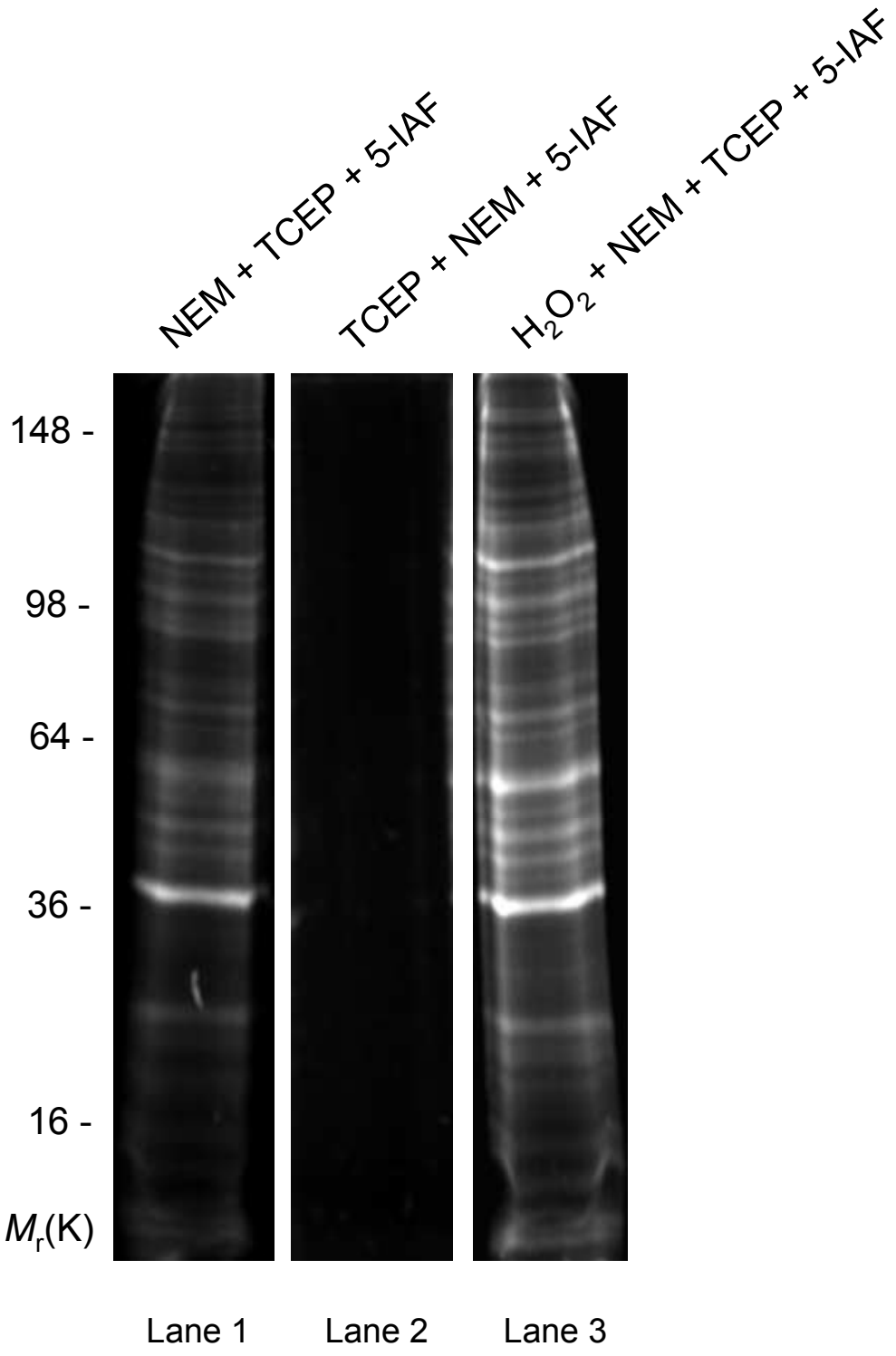

NEM + TCEP + 5-IAF
TCEP + NEM + 5-IAF
H2O2 + NEM + TCEP + 5-IAF
148 -
98 -
64 -
36 -
16 -
Mr(K)
Lane 1
Lane 2
Lane 3

Figure 5

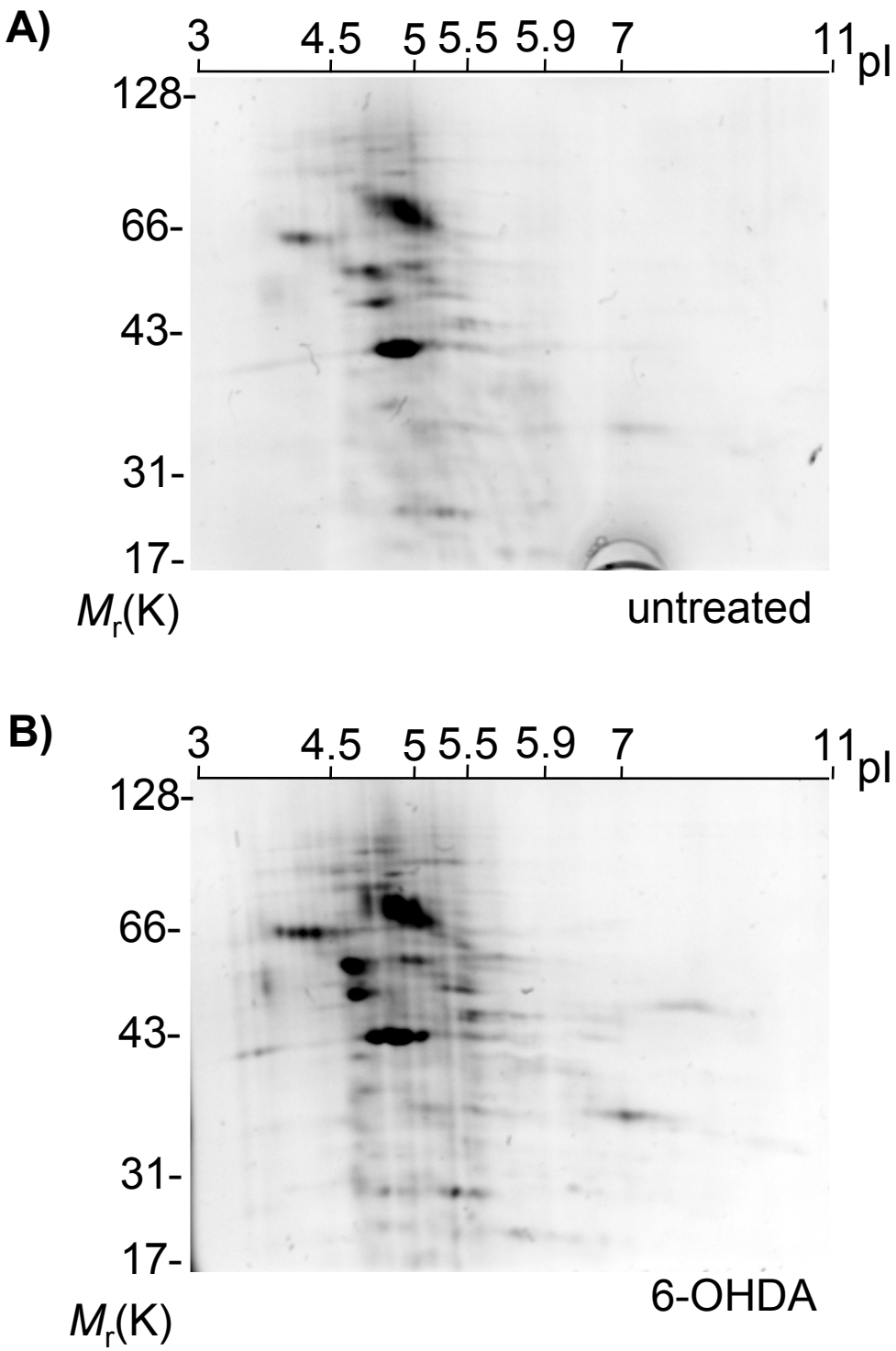

A)
3
4.5
5
5.5
5.9
7
11
pl
128-
66-
43-
31-
17-
M_r(K)
untreated
B)
3
4.5
5
5.5
5.9
7
11
pl
128-
66-
43-
31-
17-
M_r(K)
6-OHDA

Figure 6

8.2 ANEXO 2

Normas da revista Neurotoxicity Research.

Instructions for Authors

Manuscript submission

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Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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The title page should include:

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- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

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Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

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Please provide 4 to 6 keywords which can be used for indexing purposes.

Text

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- Use italics for emphasis.
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- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
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- Word template (zip, 154 kB)

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Abbreviations should be defined at first mention and used consistently thereafter.

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Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.
Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

Scientific style

- Please always use internationally accepted signs and symbols for units (SI units).

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- Please use the standard mathematical notation for formulae, symbols, etc.:

  Italic for single letters that denote mathematical constants, variables, and unknown quantities

  Roman/upright for numerals, operators, and punctuation, and commonly defined functions or abbreviations, e.g., cos, det, e or exp, lim, log, max, min, sin, tan, d (for derivative)

  Bold for vectors, tensors, and matrices.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

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The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
Reference list entries should be alphabetized by the last names of the first author of each work.

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  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

- www.issn.org/2-22661-LTWA-online.php

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- EndNote style (zip, 3 kB)

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- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

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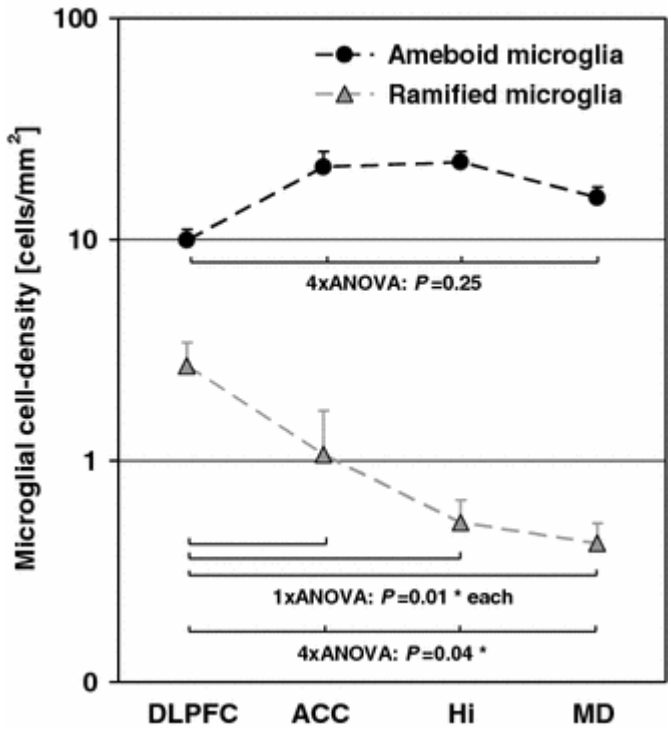

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- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
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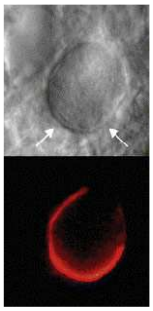

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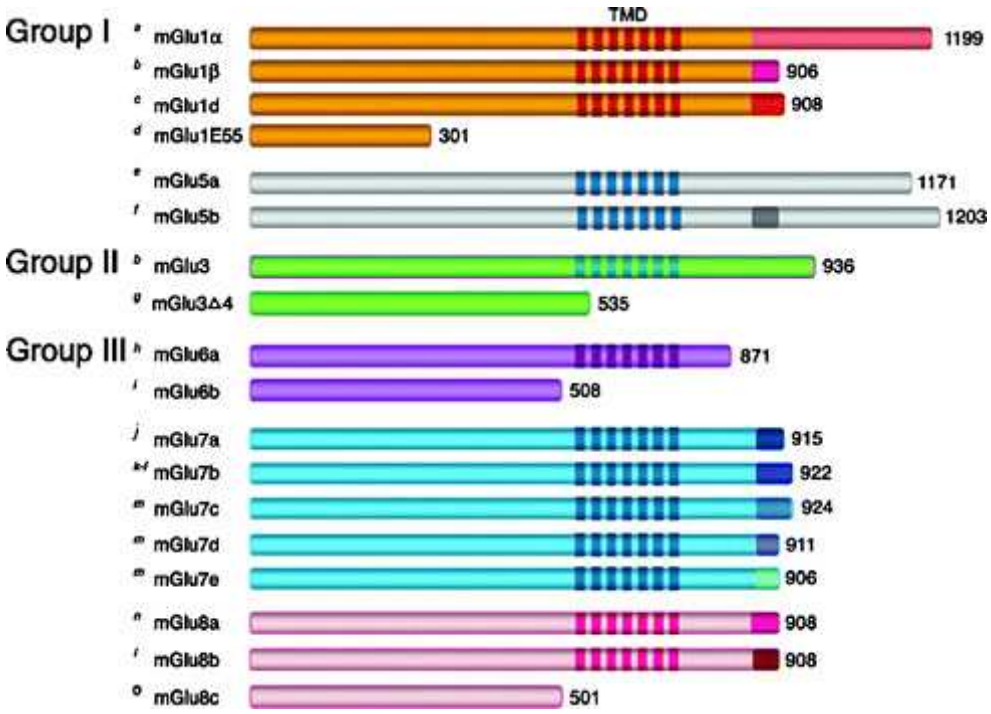

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8.3 ANEXO 3

Normas da revista The Journal of Biological Chemistry.

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The pH Dependence of Hairpin Ribozyme Catalysis Reflects Ionization of an Active Site Adenine*

Joseph W. Cottrell[1], Lincoln G. Scott[2], and Martha J. Fedor[1]

[1]From the Department of Chemical Physiology, the Department of Molecular Biology and The Skaggs Institute for Chemical Biology
The Scripps Research Institute, La Jolla, CA 92037

[2]Cassia, LLC, San Diego, CA 92109

*Running title: *Correspondence of functional and microscopic pK_a values in a ribozyme*

To whom correspondence should be addressed: Martha J. Fedor, Department of Chemical Physiology, Department of Molecular Biology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA, USA, Tel.: (858) 784-2770; Fax: (858) 784-2779; E-mail: mfedor@scripps.edu

Keywords: RNA catalysis; ribozyme; fluorescence

Background: The hairpin ribozyme is a prototype of RNA enzymes that mediate catalysis without divalent cation cofactors.
Results: Self-cleavage activity decreases as the fraction of the protonated form of an active site adenosine increases.
Conclusion: The neutral, unprotonated form of adenosine interacts with the nucleophilic- or leaving-group oxygen to facilitate catalysis.
Significance: Learning how RNA functional groups participate in catalysis is crucial for understanding RNA-mediated processes in biology.

SUMMARY

Understanding how self-cleaving ribozymes mediate catalysis is crucial in light of compelling evidence that human and bacterial gene expression can be regulated through RNA self-cleavage. The hairpin ribozyme catalyzes reversible phosphodiester bond cleavage through a mechanism that does not require divalent

Microscopic and apparent pK_a values were virtually the same, evidence that A38 protonation accounts for the decrease in catalytic activity with decreasing pH. These results implicate the neutral, unprotonated form of A38 in a transition state that involves formation of the 5'-oxygen-phosphorus bond.

Hairpin ribozymes (Hp Rzs) belong to one of several families of small self-cleaving RNAs that serve as useful models of RNA catalysis because they are relatively simple and amenable to chemogenetic analyses (1). The Hp Rz remains functional in the absence of divalent metals, relying exclusively on nucleotide functional groups for catalytic chemistry (2-5). High resolution structures of the Hp Rz bound to transition state mimics show two active site purines, G8 and A38, positioned in a similar manner to the two histidines in RNase A that mediate general acid base catalysis of the same reaction (6-8) (Fig. 1A), leading to the proposal

The text should be single-spaced with one-inch margins on the left and right sides. Once the text of the manuscript is completed in Word, convert the “Capsule/Abstract” through “Discussion” sections from a single-column format to double column format. Select those sections, click on “Format,” and then “Columns” from the drop-down menu. Select two columns and equal column width, and then change the spacing to 0.25 inches. Title and running title sections, as well as references, footnotes, figure legends and tables, should remain in single-column format.

Use 11-point Times New Roman font, and select size and bolding to mimic the appearance shown above for the title section.

Do not imbed graphics in the Word document, but add them at the end of the PDF as described below.

2. Prepare figures at publication-quality resolution, using only applications capable of generating high-resolution TIFF or EPS files. Number each figure. For important requirements for the preparation of figures, go tohttp://art.cadmus.com/da/jbc/index.jsp. An application called Rapid Inspector allows authors to check their figure files against JBC standards for format, resolution, color space and other figure requirements. Click here to download Rapid Inspector

3. Using Adobe Acrobat (see http://adobe.com/products/main.html for information), save your manuscript text and figures in a single file in PDF format with the figures at the end. The file name should be one word with no spaces and have a .pdf extension (e.g. manuscript.pdf). Authors are responsible for assuring the accuracy and quality of the PDF. Please consult your Adobe Acrobat manual for instructions regarding PDF file creation.

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5. Submit your manuscript at http://submit.jbc.org. You will need:

- Contact information for the submitting author
- Information about the authors and the manuscript
- A cover letter with information for the Editor and Associate Editors
- Single PDF file of your manuscript under 10 MB in size. **This file should contain text and figures but NOT SUPPLEMENTAL DATA.**

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Revised manuscript submission

Revised manuscripts are prepared as described above and submitted via http://submit.jbc.org/. Responses to raised concerns should be transmitted in the cover letter and not in the PDF file of the manuscript.

Report submission (formerly known as Accelerated publication)

Reports are concise but complete papers that present information on topics of exceptional novelty, significance and broad interest to readers of the JBC. To undergo expedited review and be featured as a report, the quality of an article must fall within the top 5 percent of all articles published in the journal. Reports are not simply short versions of regular papers, and the existence of competing manuscripts under concurrent review is not sufficient to merit report status. Articles that do not meet the criteria as a report may be returned to authors without a full review. Reports must be five pages or fewer, including all figures, references and tables. (A standard five-page paper contains 30,000 characters, including spaces, plus two tables or figures.)

Final submission of an accepted manuscript

If your manuscript is accepted for publication by JBC, the final PDF version will be published as one of the JBC Papers in Press within 24 hours of acceptance.

You are then required to send the final version as source files, including a Word file for text and graphic TIFF and EPS files for the figures, to Cadmus Professional Communications via its FTP site. Instructions will follow acceptance of the manuscript. Those files must be the same files from which the final PDF was prepared; otherwise, they will be rejected, delaying publication.

Source files for supplemental data are **not** needed at the time of acceptance. We will use the files included with your submission.

Organization of the manuscript

1. Formatted for standard 8.5-by-11-inch paper
2. Single spacing throughout
3. Two-column page format, including “Capsule/Abstract” through “Discussion”

sections. Title section as well as references, footnotes, figure legends and tables at the end of the manuscript are in single-column format. Click here to see an example.
4. One-inch left and right margins and 0.25-inch spacing between columns
5. Text typed in 11-point Times New Roman
6. Manuscript is to be arranged in the following order:

(a) title, author(s), complete name(s) of institution(s) and running title
(b) capsule/abstract
(c) introduction
(d) experimental procedures
(e) results
(f) discussion
(g) references
(h) footnotes
(i) figure legends
(j) tables
(k) figures
(l) supplemental data (If applicable, submit file(s) in the Supplemental Data section of the submission site.)

7. Number all pages, including those with figures. Note: Any paper submitted without page numbers will be deleted, and you will be asked to resubmit with pages numbers.

Text

Title section

It is important that the major findings of your study are intelligible to all JBC readers, including those who are not specialists in the field. The title should be as short and informative as possible (not to exceed two lines). If acronyms or abbreviations are used, the name/term should be first indicated in full followed by the short form/acronym (*e.g.*, *Visualization of Polarized Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) Activity in Live Cells by Fluorescence Resonance Energy Transfer (FRET) Imaging.*) A full name is not required for the most common biochemical abbreviations (e.g. ATP). Please review your title and abstract carefully to make sure they convey your essential points succinctly and clearly.

Authors

Contains names of all authors and their complete mailing addresses and identifies who will receive correspondence regarding the

a) name
b) telephone and fax numbers
c) e-mail address

JBC authors whose names are normally represented by non-Latin characters can now incorporate those characters into the author area of the article. Only characters that can be encoded in Unicode such as Chinese, Japanese, Korean, Cyrillic and Arabic are acceptable. These characters can only be used for author names, not author affiliations or titles.

The non-Latin characters can be added into the manuscript PDF at submission. After acceptance, the non-Latin characters should be included in the source file (Word

document). Non-Latin characters should be enclosed in parenthesis after the transliterated version (.

Running title

Will be at the top of each page and cannot exceed 60 characters and spaces

Capsule

Each paper published in the Journal of Biological Chemistry includes a brief summary known as a "Capsule." A "Capsule" reports the core findings of a paper in a way that makes clear to all JBC readers how those findings significantly advance the understanding of a biological process.

How to write the Capsule

The "Capsule" is **highly visible** component of a JBC publication so it should be crafted with **great care and precision**. Using language with which all readers are familiar will make the findings accessible to a broad scientific community and to the general public. The Capsule and the abstract have quite different purposes and audiences, so **the Capsule is not simply a truncated version of the abstract**.
A Capsule should have **60 words** or less and four parts that communicate the following to readers of all levels of interest and expertise:

1. **Background**: A complete sentence that explains the impetus and context of the work.
2. **Results:** A complete sentence that summarizes the major findings.
3. **Conclusion:** A complete sentence that summarizes the interpretation of the findings.
4. **Significance:** A complete sentence about the paper's impact on the field and its long-term implications.

After the Capsule is written, it may be helpful to do the following:

- Spell out acronyms that would not be understood by all JBC readers.
- Honestly answer this question: "Will readers walk away from what you've written with a clear understanding of what you've found and its significance?" If your answer is "no" or "maybe," it's probably best to begin again.
- Ask a nonspecialist to read your draft "Capsule" to make sure that it is clear and communicates the essence of your paper to a general reader.

Example of a Capsule

- **Background:** Metabolite binding to riboswitch RNAs regulates expression of metabolic genes.
- **Result:** Inhibitory and activating ligands interact with the same riboswitch.
- **Conclusion:** Riboswitches integrate information about the overall metabolic state of the cell.
- **Significance:** This might be the first sign of a complex RNA-metabolite interactome.

Summary

- Should succinctly and clearly describe the major findings reported in the manuscript
- Must not exceed 250 words

Introduction

- Presents the purpose of the studies reported and their relationship to earlier work in the field
- Should not be an extensive review of the literature nor, in general, exceed one typed page

Experimental procedures

- Brief but sufficiently complete to permit a qualified reader to repeat the experiments reported
- Only truly new procedures should be described in detail
- Cite previously published procedures in references
- Modifications of previously published procedures not given in detail except when necessary to repeat the work

Results

- Presented in figures and tables
- Some results not requiring documentation given solely in the text

Discussion

- Concise (usually less than two typed pages)
- Focused on the interpretation of the results rather than a repetition of the "Results" section

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References

- Cited in text by number and not by author, title, and/or date
- Titles should be included in references
- Numbered consecutively in the order of appearance
- References for journals and books should be in the following styles:

1. MacDonald, G.M., Steenhuis, J.J., and Barry, B.A. (1995) A difference Fourier transform infrared spectroscopic study of chlorophyll oxidation in hydroxylamine-treated photosystem II. *J. Biol. Chem.* **270**, 8420–8428
2. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. *References appearing as e-pubs should be in the following style*: Aphasizheva, I., Aphasizheva, R., and Simpson, L. (April 1, 2004) RNA editing terminal uridylyl transferase 1: identification of functional domains by mutational analysis. *J. Biol. Chem.* 10.1074/jbc.M401234200
4. Farrell, C. (1992) *The Role of SecB during Protein Export in Escherichia coli*. Ph.D. thesis, The Johns Hopkins University

Journal names are abbreviated according to Chemical Abstracts (http://www.cas.org/).

Authors are responsible for the accuracy of the references.

Miscellaneous

- **Footnotes** are used to cite manuscripts in preparation, unpublished observations and personal communications. Authors are responsible for obtaining written approval for all personal communications and sending a copy of the manuscript to those cited. The Editor may request proof of such approval.
- **Abbreviations** used in the text must be defined in a single footnote immediately after the first abbreviation is cited. The abbreviations of some important biochemical compounds, *e.g.* ATP, NADH, DNA, and amino acids in proteins, need not be defined. A complete list is available on the abbreviations page. Names of enzymes are usually not abbreviated except in terms of the substrates for which there are accepted abbreviations, *e.g.* ATPase and RNase.
- **The trivial and systematic names of enzymes** should be those recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) in "Enzyme Nomenclature, Recommendations, 1992*" (1992, Academic Press).*
- **Notes** added in proof to a manuscript only with the consent of the Associate Editor.
- **Errors** in a published paper will be corrected in the journal in "Additions and Corrections."

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Tables and figures

The number of tables and figures used to present data essential to illustrate or prove a point should be kept to a minimum. Very complex or large tables should be submitted as figures or as supplemental data. (For example, amino acid or nucleic acid sequences with alignments — see below).

Tables should have titles and sufficient experimental detail in a legend immediately following the title to be understandable without reference to the text. Each column in a table must have a heading, and abbreviations, when necessary, should be defined in the legend.

Figures should have titles and legends containing sufficient detail to make the figure easily understood. Legends should be organized consecutively in a separate section of

the manuscript. Authors are responsible for providing digital art that has been properly sized and cropped. Appropriately sized numbers, letters, and symbols should be used so they are no smaller than 2 mm after reduction to a single column width (21 picas, 8.9 cm, 3.5 inches), a 1.5-column width (30 picas, 12.7 cm, 5 inches), or a full two-column width (43 picas, 18.2 cm, 7.2 inches). Superscript and subscript characters are not excluded from this rule. Numbers, letters and symbols used in multipaneled figures must be consistent. The abscissa and ordinate should be clearly labeled with appropriately sized type, and units of measurement must be given. **Failure to comply with these specifications will require new figures and delay publication**. Scales for plotting the data should be marked by short index lines, but every index line need not be numbered. Use standard symbols found in Microsoft Word with symbols and curves identified in the legend and not on the figure. Indicate the figure number on each figure.

All figures should be created with applications that are capable of preparing high-resolution TIFF or EPS files acceptable for publication. Although you will initially submit figures with the text in a single PDF file, we will require submission of figures as separate TIFF or EPS files at publication-quality resolution for online publication if your paper is accepted. For important information on the preparation of figures in TIFF or EPS, go tohttp://art.cadmus.com/da/jbc/index.jsp. The list of acceptable graphic Mac OS and Microsoft Windows applications may be found at http://art.cadmus.com/da/jbc/applications.jsp. These applications can be used to successfully create high-quality TIFF and EPS files, and you will find instructions on how to save them properly. Because more applications are added as testing continues, please review this list periodically. It is the author's responsibility to verify the quality of the graphics in the PDF that has been prepared and that compression of the files for submission does not distort the images.

While image manipulation is often desirable for clarity and/or brevity of presentation, manipulation for deceptive purposes either to unfairly enhance or eliminate or otherwise obscure data is misconduct and will be resolved according to JBC policy.

For graphic material, we have adopted a policy taken from The Journal of Cell Biology.
"No specific feature within an image may be enhanced, obscured, moved, removed, or introduced. The groupings of images from different parts of the same gel, or from different gels, fields or exposures must be made explicit by the arrangement of the figure (e.g. using dividing lines) and in the text of the figure legend. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend."

Please be aware of several key issues when preparing graphics for publication:

1.The JBC is now in an RGB (Red, Green, Blue) workflow for color figures.Prior to June 1, 2005, authors were required to submit figures in CMYK (Cyan, Magenta, Yellow, blacK) color mode, as this is the native color mode for the printing process and thus optimizes color for press. Today, all color figures should be submitted in RGB format to retain the brilliant reds, greens and blues for online publication. To learn more, please see http://art.cadmus.com/da/jbc/index.jsp.
2.Images supplied in EPS format should have all fonts converted to outlines/paths.If fonts are not converted to paths or outlines, there is a possibility of character substitutions or that your graphic may have to be converted to a bitmap, which can affect online image quality.

The following screenshot demonstrates how to convert fonts to outlines/paths in Adobe Illustrator:

3. Excessive file compression for submission can distort images, so PDFs should be carefully checked.
4. Images containing both grayscale and bitmap areas must be supplied at a minimum resolution of 600 dpi. If possible, it is best to prepare such images as vector files for the line art areas, with embedded TIFF images for the grayscale portions.
5. We cannot accept figure files in certain applications, such as Microsoft Office (PowerPoint, Word, Excel, Access), Corel Perfect Office (WordPerfect, Quattro Pro, Presentations), and Lotus SmartSuite (Freelance Graphics, 1-2-3, Approach, and WordPro). They are not intended for high-resolution imaging necessary for publication. For example, problems with PowerPoint that affect both the visual quality and accuracy of print reproduction include:
Poor color reproduction: PowerPoint files are intended for screen display (RGB color model). Colors that are represented in a PowerPoint presentation may not be possible to reproduce on printing.
Lack of resolution control: PowerPoint is intended for screen displays rather than print production, so it does not offer full control over resolution. Lettering and other figure parts may appear jagged.
Poor font management: If a computer does not have a font in a PowerPoint presentation created on another computer, it will substitute the font (usually with nonsense) without warning.

To learn how to convert PowerPoint images to an acceptable format for publication, please see https://rapidsubmission.cadmus.com/jbc/JBC%20PowerPoint%20Update.pdf

Below are examples of a good-quality figure and a poor-quality figure. The good-quality figure has all the wording on the figure in proportion, whereas the poor-quality figure has a wide variety of lettering sizes.

After a manuscript is judged acceptable for publication by JBC, the figure TIFF and EPS files as well as the Word text file will be submitted online at a FTP site maintained by Cadmus Professional Communications. Specific instructions will follow acceptance of the manuscript.

There will be a no charge for publication of halftone figures. We encourage authors to use color figures when they will enhance the presentation of the data. The cost is $150 for each illustration containing color. (Note: When using the electronic submission system, any figure submitted in color will be reviewed and processed with the understanding that the figure will be published in color.)

If you require further information, please contact jbc@asbmb.org

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Supplemental data

Manuscripts must be complete, stand-alone, and NOT dependent on supplemental data. The JBC Online does, however, allow authors to include supplemental (or supporting) data. This includes data that would be important and useful to specialists as exemplified by chemical structures, spectra, and kinetic plots that are not critical to the manuscript. Links to author or third-party sites for such information are not allowed because they lack permanence, though links to “official” databases like GenBank are encouraged.

We encourage authors to include such things as videos, long lists of primers, 3-D structures/images (though it is now possible to include 3-D PDFs in the manuscript), sequence alignments, and data sets that are very large, such as those obtained with microarray hybridization experiments or mass spectrometry studies.

Supplemental data will be reviewed as a part of the normal manuscript review process and will be judged by the same rigorous criteria to be important but not essential to the stand-alone manuscript. Only data that are deemed appropriate for the online journal and substantially contribute to the manuscript will be accepted. Supplemental data submitted during review will require that the paper be reviewed again thus extending the review process. Supplemental data submitted after the paper has been accepted will not be published.

Supplemental Data should NOT contain preliminary data that simply extends the scope of the study, unnecessary "control" data, or data that are thought to be not rigorous enough for the main text. Some novel methodology may be presented in detail in the Supplemental Data, but it should not be viewed as a "depository" for most methods; the main text should contain sufficient methodology for an experienced investigator to replicate the experiments. Authors should carefully review the Supplementary Data for factual, grammatical, and typographical issues since this material will not be professionally copyedited but permanently posted "as is."

All compatible data files MUST BE combined into a single PDF for submission. Movies and large Excel files should be submitted in their native formats.

3-D images embedded in PDFs are accepted as part of manuscripts. Please alert the JBC office by email at the time of submission (this option will be offered to you) if you plan on submitting a 3-D image so that the files can be handled properly. If the file size is too large for the system due to a 3-D image, ASBMB staff will allow the file through. For more information on how to create 3-D files for submission, see this tutorial or read the how-to transcript.

Source files for supplemental data are **NOT** needed at the time of acceptance. We will use the files included in your submission. Supplemental files cannot be added after acceptance.

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Publication charges

Page charges: $80 per journal page for the first nine pages; $160 per page starting with the 10th page
Color figures: We encourage authors to use color figures when they will enhance the presentation of the data. The cost is $100 for each illustration containing color. (*Note*: When using the electronic submission system, any figure submitted in color will be reviewed and processed with the understanding that the figure will be published in color.)
Discounts on Publication Fees: Regular ASBMB members who publish as the corresponding author in JBC will receive discounts of $10 per page and $50 per color figures, saving an average of $240 per publication.
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Chemical and mathematical usage

Numerical data should be reported with the number of significant digits that corresponds to the magnitude of experimental uncertainty. Table I lists the abbreviations for units of measurement and certain physical and chemical quantities used by the JBC without definition. Also listed are the prefixes that can be added to names of units and the multipliers indicated by each prefix.

Chemical equations, structural formulas and mathematical equations should be placed between successive lines of text. In general, the rules and recommendations of the IUBMB and the International Union of Pure and Applied Chemistry (IUPAC) will be used for abbreviation of chemical names, nomenclature of chemical compounds, enzyme nomenclature, isotopic compounds, optically active isomers, and spectroscopic data. We recommend the use of the classification, nomenclature, and structural representation of lipids used by the LIPID MAPS Initiative (see Fahy et al. *J. Lipid Res.* 2005 46: 839–862). You can download lipid structures directly from the LIPID MAPS Structure Database (http://www.lipidmaps.org). This database draws structures de novo, allowing you to insert them into your documents. Table II lists references to publications of the rules and recommendations of the International Scientific Unions that may be consulted for detailed information.

Enzyme activity data

Papers reporting kinetic and thermodynamic data concerning enzymes and other catalytic proteins and nucleic acids should include the identity of the enzymes, additional biological information (e.g. species and tissue normally found in, post-translational modification), preparation and criteria of purity, assay conditions, methodology, activity, and any other information relevant to judging the reproducibility of the results. See the Beilstein Institut/STRENDA (standards for reporting enzymology data) commission Web site (http://www.strenda.org/documents.html) for more details and suggestions.
Enzyme activity (steady-state) generally should be reported in terms of V_{max} (nmol or μmol product formed per amount ((protein)) per time) or, when possible, as k_{cat} (V_{max} divided by molar enzyme concentration), in min^{-1} or s^{-1}. K_m units are given in molarity.

Any other units of activity (absorbance, % change) should be converted to units of molarity to express k_{cat} or V_{max}. Values of k_{cat} (V_{max}) and K_m should be estimated using nonlinear fitting (and the software system cited).

Parameters should include estimates of error (e.g. SE). The use of linear transformation for Michaelis-Menten parameters is recognized to be inaccurate and use of an alternate method should be justified (e.g., graphical presentation of inhibition).

A lack of activity should be defined in terms of a limit of detection. In a series of comparisons to a basal or "control" level of activity (e.g., set as unity or "100%"), this activity should be indicated, in the units mentioned above, along with estimates of error.

The inclusion of examples of some of the raw data is encouraged, at least as part of a Supplemental Data section. Please refer to the STRENDA Web site regarding enzyme inhibition (http://www.strenda.org/documents.html). $K_i$values are preferred to IC_{50}.

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Protein and nucleic acid sequences

Newly reported nucleotide or protein sequences must be deposited in GenBank or EMBL databases, and an accession number must be obtained before the paper is accepted by the Associate Editor. Access to the information in the database must be available at the time of publication.

Authors are responsible for arranging release of data at the time of publication. The authors also must provide a statement in the manuscript that this sequence has been scanned against the database and all sequences with significant relatedness to the new sequence identified (and their accession numbers included).

Authors of accepted papers containing nucleotide sequences must submit the sequence data, preferably in computer-readable form or by e-mail, and a copy of the paper to one of the following:

GenBank Submissions

National Center for Biotechnology Information
8600 Rockville Pike, Building 38A
Room 8N-805
Bethesda, MD 20894
Phone: 301-496-2475
http://www.ncbi.nlm.nih.gov/Genbank/index.html

Or

EMBL Nucleotide Sequence Submissions

European Bioinformatics Institute
Hinxton Hall
Hinxton, Cambridge CB10 1SD, UK
Phone: 44-1223-494401
Fax: 44-1223-494472
E-mail: support@ebi.ac.uk
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Or

DNA Data Bank of Japan

Center for Information Biology
National Institute of Genetics
Mishima, Shizuoka, 411, Japan
Phone: 81-559-81-6853
Fax: 81-559-81-6849
http://www.ddbj.nig.ac.jp

A footnote will be included in the paper indicating that such a deposit has been made. Submission to any data bank is sufficient to ensure entry in all. When nucleotide probes are used, the ends of the probes should be explicitly identified by reference to published nucleotide number or restriction maps, or, if unpublished, the information should be included in the "Experimental Procedures" section.

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Genomic and proteomic studies

Authors of papers that include genomic, proteomic or other high-throughput data are required to submit their data to the NCBI gene expression and hybridization array data repository (GEO, http://www.ncbi.nlm.nih.gov/geo/) and to provide the GEO accession number. The data must be submitted and an accession number obtained before the Associate Editor accepts the paper. Release of the information in the database must be available at the time of acceptance, because they are published immediately as Papers in Press. Access to the information in the database must be available at the time of publication.
GEO has a Web-based submission route, suitable for a small number of samples, or a batch submission tool (called SOFT). GEO is accessible
from http://www.ncbi.nlm.nih.gov/geo/.
Submission frequently asked questions are
at http://www.ncbi.nlm.nih.gov/geo/info/general_faq.cgi.
Submitted data is encouraged to follow the MIAME checklist. For more information, seehttp://www.mged.org/Workgroups/MIAME/miame.html.

Proteomic Data

The JBC requires that authors of manuscripts

1. Document in the methods section the mass spectrometers and experimental protocols used for peptide/protein analysis, as well as the programs used and the size and version details of the sequences databases used for matching peak lists to protein or peptide sequences. This should include details of the mass tolerances allowed in the matching process and explicitly provide thresholds and values specific to judging the certainty of each identification. The authors should explain any statistical analysis applied to validate the results and provide a determination of false-positive detection rates for large-scale experiments.

2. Justify any conclusions citing quantitative proteomic results with how the biological reliability of measurements was validated using biological replicates, statistical

methods and independent experiments, and provide proper estimates of uncertainty and the methods used for the error analysis.

3. Provide as supplemental data: tables of accession numbers, score(s) and any associated statistical information obtained from searches conducted. This should include sequence coverage and the total number of nonredundant peptides assigned to the protein.

4. Provide as supplemental data: annotated mass spectra when post-translational modifications are claimed or single-peptide identifications are critical for the conclusions of the manuscript.

The JBC considers the recommendations (http://mcponline.org/site/misc/ParisReport_Final.xhtml) and checklist (http://www.mcponline.org/site/misc/CheckList.pdf) of Molecular & Cellular Proteomics a helpful guide for authors of manuscripts containing proteomic data.

The JBC encourages submission of MS/MS spectral data by the authors to group Web sites and/or public repositories for access by both reviewers and readers.

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Structural studies

For papers describing structures of biological macromolecules, the atomic coordinates and the related experimental data (structure factor amplitudes/intensities and/or NMR restraints and chemical shifts) must be deposited at a member site of the Worldwide Protein Data Bank (wwpdb.org; info@wwpdb.org), RCSB PDB (rcsb.org), PDBe (pdbe.org) PDBj (www.pdbj.org) or BMRB (www.bmrb.wisc.edu).

The PDB ID assigned after deposition should be included in the manuscript. Authors must also submit the PDB Validation Report (provided after annotation by the wwPDB) to JBC for review.

PDB data must be released before final acceptance of the manuscript. No data is to be withdrawn from PDB once a paper has been accepted and published as a Papers in Press article. As of December 2006, PDB no longer accepts coordinates for model structures determined by computational methods. The coordinates must be included as a supplement to the online paper and formatted just as if it were a PDB submission.

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Database accession hyperlinks for JBC Online

The electronic version of the journal employs direct hyperlink access to entries in databases like GenBank. Authors must provide accession numbers to databases for all newly described molecular structures reported in their manuscripts.

In addition, authors are strongly encouraged to include accession numbers for any database information that would aid a reader in understanding the paper, regardless of who deposited the database information.

For database hyperlinks to be generated, the citation must appear as a footnote and be written as follows:

1. GenBank = GenBank Accession Number XYYYYY
2. Molecular Modeling Database = MMDB # XYYY
3. NCBI Protein Database = NCB Accession # XXXXX
4. Swiss Protein Database = Swiss-Prot # XXXX
5. Enzyme Collection Number = xx.yy.zz.bb
6. Research Collaboratory for Structural Bioinformatics Protein Databank = PDB # XXXX

The molecule or structure for an accession number can be identified through the NCBI Entrez utility at http://www.ncbi.nlm.nih.gov/Entrez/.

The following shows how references to databases should be written in a footnote:

The nucleotide sequence for the artificial sperm whale myoglobin gene has been deposited in the GenBank database under GenBankAccession Number (Reference). The amino acid sequence of this protein can be accessed through NCBI Protein Database under NCBI Accession # 2311060 (Reference). The atomic coordinates for the crystal structure of this protein are available in the Molecular Modeling Database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=structure) under MMDB # 5MBN(Reference).

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Authors may include their e-mail addresses and homepage URLs. However, authors cannot cite in the manuscript that additional data not presented in the manuscript are available on the Web. Homepage URLs will not be hyperlinked.

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