Universidade Federal do Rio Grande do Sul

EFEITOS DA APOMORFINA E DE UM PRODUTO DERIVADO DE SUA AUTOXIDAÇÃO EM DIFERENTES MODELOS BIOLÓGICOS

Jaqueline Nascimento Picada

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Orientador: Prof. Dr. João Antonio Pêgas Henriques

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Este trabalho apresenta-se organizado da seguinte forma: a introdução aborda os aspectos mais relevantes da apomorfina, como suas atividades pró-/antioxidante, neuroprotetora e suas atividades farmacológicas. Também são apresentados na introdução todos os experimentos que levaram ao corpo de resultados distribuídos nos capítulos I, II, III e IV. Uma discussão geral busca integrar os achados descritos nos capítulos anteriores. Na seqüência, são descritas as conclusões e as perspectivas geradas por este trabalho. Em anexo, consta o artigo "Dose finding in the Ames *Salmonella* assay", no qual o produto de oxidação de apomorfina, 8-oxo-apomorfina-semiquinona foi abordado pela primeira vez.

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ABREVIATURAS

APO	apomorfina
CAT	catalase
CHL	Células de pulmão de hamster Chinês (“Chinese hamster lung cells”)
D1/D2	receptor de dopamina do tipo D1 e D2
DA	dopamina
DP	Doença de Parkinson
EC	Ensaio Cometa
EI	Esquiva Inibitória
ERO	espécies reativas de oxigênio
Gpx	glutationa peroxidase
GSH	glutationa reduzida
GSSG	glutationa oxidada
H_2O_2	peróxido de hidrogênio
IL	interleucina
LTM	memória de longa duração (“Long-term memory”)
MAO-B	monoaminoxidase do tipo B
MPTP	N-metil-4-fenil-1,2,3,6-tetrahidropiridina
NF-κB	fator de transcrição NFκB
NMDA	N-metil-D-aspartato
$O_2^{\bullet-}$	radical superóxido
8-OASQ	8-oxo-apomorfina-semiquinona
6-OHDA	6-hidroxidopamina
OxyR	fator de transcrição OxyR
PC12	Células feocromocitoma 12 (“pheochromocytoma cells 12”)
SOD	superóxido dismutase
STM	memória de curta duração (“Short-term memory”)
t-BOOH	terc-butil-hidroperóxido
TH	Tirosina hidroxilase
Yap1	fator de transcrição yap1
YDP	meio completo para leveduras

RESUMO

Apomorfina é um potente agonista dopaminérgico D_1/D_2, utilizada no tratamento da Doença de Parkinson. Em maio de 2001, apomorfina HCl foi aprovada para utilização no tratamento da disfunção erétil, aumentando o número de usuários potenciais deste fármaco. Estudos sugerem que apomorfina e outros agonistas dopaminérgicos podem induzir neurotoxicidade mediada por seus derivados de oxidação semiquinonas e quinonas, os quais levam à formação de espécies reativas de oxigênio.

Os objetivos do presente estudo foram de avaliar os possíveis efeitos genotóxicos, antimutagênicos, citotóxicos de apomorfina (APO) e de um produto derivado de sua oxidação, 8-oxo-apomorfina-semiquinona (8-OASQ), utilizando o teste *Salmonella*/microssoma, Mutoxiteste WP2, ensaio Cometa e teste de sensibilidade em *Saccharomyces cerevisiae*. Em adição, foram avaliados os efeitos de APO e 8-OASQ sobre a memória e o comportamento em ratos (tarefa de esquiva inibitória, comportamento e habituação ao campo aberto) e o comportamento estereotipado em camundongos.

Ambos compostos induziram mutações por erro no quadro de leitura em linhagens de *S. typhimurium* TA97 e TA98, sendo que 8-OASQ foi cerca de duas vezes mais mutagênico que APO, na ausência de S9 mix. Para linhagens que detectam mutágenos oxidantes, 8-OASQ foi mutagênico, enquanto APO foi antimutagênico, inibindo a mutagenicidade induzida por H_2O_2 e t-BOOH em linhagens de *S. typhimurium* e derivadas WP2 de *E. coli*. O S9 mix inibiu todos os efeitos mutagênicos, provavelmente retardando a oxidação de APO ou devido à conjugação de APO e seus produtos de autoxidação, como 8-OASQ, a proteínas do S9. Em testes de sensibilidade

com *S. cerevisiae*, APO foi citotóxica para algumas linhagens apenas nas doses mais altas. Para 8-OASQ este efeito foi dose-depende para todas as linhagens, sendo que as mutantes deficientes em catalase (*ctt1*), superóxido dismutase (*sod1*) e *yap1* foram as mais sensíveis. APO protegeu as linhagens de *S. cerevisiae* contra danos oxidativos induzidos por concentrações altas de H_2O_2 e t-BOOH, enquanto que 8-OASQ aumentou os efeitos pró-oxidantes e induziu respostas adaptativas para aqueles agentes.

APO e 8-OASQ induziram efeitos de prejuízo na memória de curta e de longa duração em uma tarefa de esquiva inibitória em ratos. APO, mas não 8-OASQ, prejudicou a habituação a um novo ambiente de forma dose-dependente. Os efeitos de prejuízo de memória não foram atribuídos à redução da nocicepção ou outra alteração inespecífica de comportamento, visto que nem APO e nem 8-OASQ afetaram a reatividade ao choque nas patas e comportamento durante a exploração ao campo aberto. Os resultados sugerem, portanto, que os produtos de oxidação de dopamina ou de agonistas dopaminérgicos podem induzir deficiências cognitivas.

APO, mas não 8-OASQ, induziu comportamento estereotipado em camundongos machos CF-1. A falta da indução deste comportamento por 8-OASQ sugere que a autoxidação de APO causa a perda na sua habilidade de ligar-se a receptores dopaminérgicos.

Pelo ensaio Cometa, 8-OASQ provocou danos ao DNA do tecido cerebral de camundongos sacrificados 1 h e 3 h, mas não 24 h após sua administração, enquanto que APO induziu um fraco aumento da freqüência de dano ao DNA 3 h após o tratamento. Esses resultados sugerem que ambos APO e 8-OASQ desempenham uma atividade genotóxica no tecido cerebral.

ABSTRACT

Apomorphine is a potent dopamine D_1/D_2 receptor agonist, which has been used in the therapy of Parkinson's disease. In May of 2001 apomorphine HCl was approved for the treatment of erectile dysfunction, increasing significantly the number of potential users of this drug. It has been proposed that apomorphine and other dopamine agonists might induce neurotoxicity mediated by their quinone and semiquinone oxidation derivatives, which lead to the formation of reactive oxygen species. Previous studies demonstrated that this compound acts either as antioxidant or as pro-oxidant.

The aim of the present study was to evaluate the possible genotoxicity, antimutagenicity, cytotoxicity, and neurobehavioral effects of apomorphine (APO) and its oxidation derivative, 8-oxo-apomorphine-semiquinone (8-OASQ), by means of the *Salmonella*/microsome assay, WP2 Mutoxitest, sensitivity assay in *Saccharomyces cerevisiae*, behavioral procedures (inhibitory avoidance task, open field behavior, and habituation) in rats and stereotyped behavior and comet assay in mice.

Both compounds induced frameshift mutation in TA98 and TA97 *S. typhimurium* strains, with 8-OASQ being up to two times more mutagenic, in the absence of S9 mix. However for strains which detect oxidative mutagens, 8-OASQ acted as a mutagen while APO was an antimutagen, inhibiting H_2O_2 and t-BOOH-induced mutagenicity in TA102 *S. typhimurium* and WP2-derived *E. coli* strains. The S9 mix inhibited all mutagenic effects, probably either by retarding oxidation of APO or by conjugation of APO and its autoxidation products, such as 8-OASQ, to proteins. In sensitivity assays with *S. cerevisiae*, APO was only clearly cytotoxic to some strains at higher doses, whereas 8-OASQ dose-dependently sensitized all the strains, mainly the catalase (*ctt1*), superoxide dismutase (*sod1*) and *yap1* deletion mutants, suggesting that

8-OASQ cytotoxicity towards *S. cerevisiae* results from its pro-oxidant properties. APO also tended to protect *S. cerevisiae* strains against oxidative damage induced by high concentrations of H_2O_2 and t-BOOH, while 8-OASQ enhanced pro-oxidant effects and induced adaptation responses to these agents.

APO and 8-OASQ induced differential impairing effects on short- and long-term retention of an inhibitory avoidance task in rats. APO, but not 8-OASQ dose-dependently impaired habituation to a novel environment. The memory-impairing effects could not be attributed to reduced nociception or other nonspecific behavioral alterations, since neither APO nor 8-OASQ affected footshock reactivity or behavior during exploration of an open field. The results suggest that oxidation products of dopamine or dopamine receptor agonists might induce cognitive deficits.

APO dose-dependently induced stereotypy in male CF-1 mice, whereas animals treated with 8-OASQ failed to show stereotypy, suggesting that autoxidation of APO causes o loss in its ability to bind dopamine receptors.

In order to investigate whether APO and 8-OASQ can induce DNA damage in mice brain, we utilized the Comet assay. 8-OASQ induced DNA damage in the brains at 1 h and 3 h, but not at 24 h after treatment whereas APO induced a slight increase in brain DNA damage frequency at 3 h after treatment, suggesting that both drugs display a genotoxic activity in brain tissue.

I. INTRODUÇÃO

1. APOMORFINA

Apomorfina, APO (4*H*-Dibenzo[*de,g*]quinoline-10,11-diol, 5,6,6a,7-tetrahydro-6-methyl-(*R*), Figura 1) é um alcalóide semi-sintético benzilisoquinolínico que foi obtido pela primeira vez em 1869 através do tratamento ácido da morfina (Neumeyer, 1985). Sua estrutura bifenílica apresenta no anel aromático D, duas hidroxilas vicinais no carbono 10 (C-10) e carbono 11 (C-11), formando um anel catecólico (Figura 1) (Cassels et al., 1995). A estrutura catecólica, juntamente com o nitrogênio metilado pertencente ao anel B, confere à apomorfina similaridade estrutural com a dopamina (Cannon et al., 1972).

Devido a estas características estruturais, APO é um agonista dopaminérgico D1/D2, que prontamente entra no cérebro e se acumula no estriado (núcleos caudado e putâmen) (Bianchi e Landi, 1985; Deleu et al., 2002). APO apresenta propriedade antiparkinsoniana similar à de L-DOPA, sendo útil no tratamento de pacientes com Doença de Parkinson (DP), especialmente nos estágios finais da doença (Sit, 2000; Deleu et al., 2002; Bonuccelli et al., 2002).

Apesar da etiologia da DP permanecer ainda incerta, esta doença é caracterizada por uma gradual degeneração de neurônios dopaminérgicos na *pars compacta* da substância negra (Duvoisin, 1991; Youdim e Riederer, 1997; Dunnett e Björklund 1999). A perda de neurônios dopaminérgicos resulta numa associada redução em fibras aferentes da substância negra para o estriado, acompanhada de uma série de desordens sensoriais e motoras, incluindo lentidão de movimentos (bradicinesia), dificuldade em iniciar movimentos voluntários (acinesia), aumento do tônus muscular (rigidez) e tremores das mãos e da mandíbula (Corboy et al., 1995; Youdim e Riederer, 1997).

Embora os principais sintomas clínicos da DP sejam as perturbações motoras (Duvoisin, 1991; Jenner e Brin, 1998), estudos em animais sugerem que a DP também envolve déficits de aprendizado e memória (Brown et al., 1997; Da Cunha et al., 2001). Pacientes com PD mostram déficits de memória (Dubois e Pillon, 1997; Goldman et al., 1998).

Muitos estudos indicam o envolvimento da produção excessiva de espécies reativas de oxigênio (ERO) na degeneração progressiva dos neurônios dopaminérgicos na DP (Dunnett e Björklund, 1999; Gerlach et al., 2000; Chiueh et al., 2000). A hipótese do envolvimento de ERO surgiu da noção de que o metabolismo da dopamina (DA) (Figura 1), por um processo químico ou enzimático, pode gerar ERO (Bindolli et al., 1992; Youdim et al., 1999; Díaz-Véliz et al., 2002). A autoxidação da DA e seu precursor L-DOPA, o qual é usado no tratamento de DP, leva a espécies quinonas potencialmente tóxicas e a ERO, enquanto a oxidação enzimática da dopamina pela monoaminoxidase tipo B (MAO- B) leva à formação de peróxido de hidrogênio (H_2O_2) que pode reagir com ferro para formar o radical hidroxil ($OH^{\bullet}$) altamente reativo, via reação de Fenton (ver item 2) (Spencer et al., 1994; Gerlach et al., 2000; Wu et al., 2000).

Recentemente APO foi aprovada para uso em tratamento de disfunção erétil, o que vem ampliando muito sua utilização clínica (Dula et al., 2000; Andersson, 2001). Também neste caso o mecanismo de ação da APO está relacionado à sua atividade dopaminérgica central, associado à ativação da via ocitocinérgica, particularmente sobre os núcleos periventriculares do hipotalálamo (revisado em Heaton, 2000).

As vias mais freqüentes para a administração de APO são a sublingual e a subcutânea, evitando o acentuado metabolismo hepático de primeira passagem, que

ocorre na via enteral (oral) (Deleu et al., 2002). Após administração, APO é rapidamente absorvida, atingindo um pico de concentração plasmática em 40-60 minutos, sendo metabolizada principalmente no fígado, formando conjugados com ácido glucurônico e sulfato (Deleu et al., 2002; Keski-Hynnila et al., 2002; Vietri et al., 2002). APO é também metabolizada por N-demetilação, levando à formação de norapomorfina e por autoxidação que gera semiquinonas e quinonas (El-Bachá et al., 2000; Deleu et al., 2002; Garrido et al., 2002).

Assim como nas catecolaminas (como a DA), o processo de autoxidação da APO gera ERO, radicais *o*-semiquinona e quinona, através da perda de elétrons pertencente ao grupamento catecólico (Cheng et al., 1979; Kalyanaraman et al., 1985; Mosca et al., 1998). Este processo oxidativo está intimamente ligado ao pH do meio, pois é necessário que ocorra primeiramente a ionização da estrutura catecólica, para depois haver a transferência eletrônica (oxidação) (Kalyanaraman et al., 1985; Garrido et al., 2002). Portanto, o processo de autoxidação da APO ocorre principalmente em pH neutro e levemente alcalino, pois em pH ácido este alcalóide adquire estabilidade devido à não dissociação das hidroxilas do grupamento catecólico (Cheng et al., 1979; Kalyanaraman et al., 1985; Mosca et al., 1998).

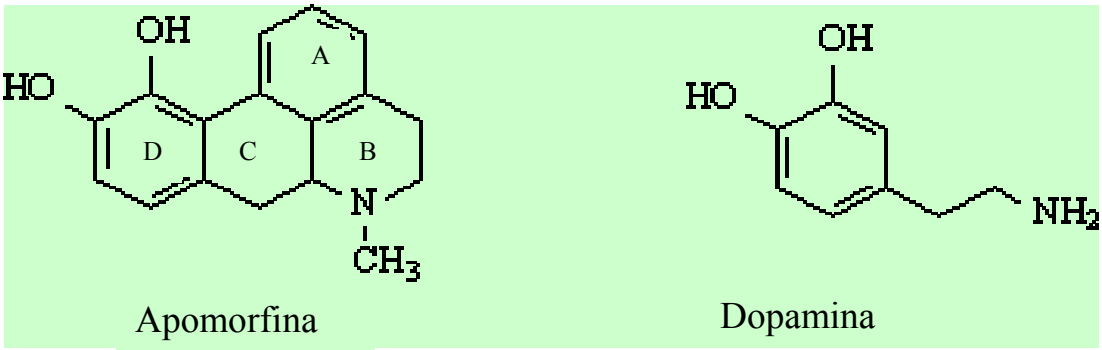

Figura1. Estrutura química da apomorfina e da dopamina.

1.1 Atividades genotóxicas, pró- e antioxidantes da apomorfina

A correlação entre mecanismos de autoxidação e ação tóxica da APO ainda não está totalmente esclarecida. Alguns estudos referentes aos efeitos deletérios provocados pela APO em sistemas biológicos têm demonstrado que o assunto é controverso. A natureza redutora natural de catecóis pode em parte explicar algumas atividades protetoras da APO contra danos oxidativos em modelos *in vitro* e *in vivo*. Por outro lado, os metabólitos oxidados derivados de APO e suas formas oxidadas podem ter atividades genotóxicas (revisado em Sit, 2000).

Suter e Matter-Jaeger (1984) verificaram que APO apresenta atividade genotóxica em bactéria devido à sua autoxidação, gerando produtos que induziram mutações por erro no quadro de leitura do DNA (mutações "frameshift") em *Salmonella typhimurium*. APO apresenta também atividade clastogênica em células de pulmão de hamster chinês (CHL), na ausência de ativação metabólica (S9 mix) (Tadaki et al., 1992). Ubeda et al. (1993) demonstraram que APO é capaz de estimular danos ao DNA induzidos por Cu^{2+} e degradação de deoxirribose induzida por Fe^{3+}, desta forma agindo como pró-oxidante. Por outro lado, este alcalóide mostrou efeito inibitório da peroxidação lipídica induzida por Fe^{3+}, apresentando, neste caso, propriedades antioxidantes (Martinez et al., 1992; Ubeda et al., 1993).

APO inibe a peroxidação lipídica em fração mitocondrial de cérebro de rato, funcionando como um potente quelante de ferro e seqüestrador de radical (Gassen et al., 1996). APO, dependendo da concentração, protege células feocromocitoma de rato (PC12) dos efeitos tóxicos de H_2O_2 e 6-hidroxidopamina (6-OHDA) e da ação do ferro. Porém, a medida que a concentração de APO aumenta, um efeito citotóxico é

observado, causando morte celular, potencializada pela presença de peróxido de hidrogênio (Gassen et. al., 1998).

Recentemente, El-Bachá et al. (2001) demonstraram que APO promove necrose em células C6 de glioma de rato e este efeito está relacionado à formação de ERO, quinonas e a um pigmento tipo-melanina durante a autoxidação da APO. As células tratadas com APO perdem a integridade da membrana e apresentam degeneração de organelas, especialmente mitocôndria e desintegração de cromatina. Tanto a autoxidação da APO quanto os danos celulares são prevenidos por compostos contendo grupo tiol. Todas as proteínas aparecem de cor verde após a incubação com APO, sugerindo a formação de um conjugado apomorfina-proteína não específico pela ligação de APO a proteínas intracelulares (El-Bachá et al., 1999; 2001).

Neuroproteção induzida por APO tem sido documentada em estudos que utilizam modelos experimentais de doenças crônicas degenerativas, incluindo os modelos de indução de Parkinsonismo MPTP (N-metil-4fenil-1,2,3,6-tetraidropiridina) e 6-OHDA (Grunblatt et al., 2000). A neurotoxina 6-OHDA, induz o fator de transcrição NF-κB redox-sensível, que ativa os promotores de vários genes envolvidos na resposta inflamatória, como as citoquinas citotóxicas (fator de necrose tumoral, TNF-α e interleucinas, IL-1 e IL-6), no corpo estriado de ratos (Grunblatt et al., 2000). Esta indução pode ser bloqueada pelo pré-tratamento com APO (10 mg/Kg, i.p.) (Youdim et al., 1999). APO previne a diminuição dos níveis de DA estriatal e de tirosina hidroxilase (TH) induzida por MPTP, uma neurotoxina que estimula a produção de ERO e contribui para a morte dos neurônios dopaminérgicos (Fornai et al., 2001; Battaglia et al., 2002a). Em particular, recentes evidências bioquímicas e moleculares indicam que a progressão dos danos neuronais observados em pacientes com DP, e que são reproduzidos nos

modelos experimentais, é devido principalmente à geração de ERO (Fahn e Cohen 1992; Ebadi et al., 1996; Grunblatt et al., 2000). Por outro lado, a neuroproteção de APO parece ser principalmente devido a suas propriedades antioxidantes, como seqüestradora de radicais livres e quelante de ferro, e é independente de sua atividade agonista dopaminérgica (Gassen et al., 1999; Youdim et al., 2000; Chen et al., 2001; Fornai et al., 2001; Grunblatt et al., 2001a; Grunblatt et al., 2001b).

1.2 Apomorfina em estudos de memória e comportamento em animais

Alterações nos diferentes tipos de memória podem ocorrer em distúrbios neurodegenerativos e/ou psiquiátricos, que estão associados aos neurotransmissores monoaminérgicos. A noradrenalina e também a serotonina estão implicadas nas doenças depressivas conforme prediz a "teoria monoaminérgica da depressão" enquanto que a dopamina está associada aos transtornos esquizofrênicos e à DP. O sistema monoaminérgico do cérebro controla várias funções como locomoção, emoção e liberação de hormônios (Smythies, 1997; Stevens, 1998; Shansis e Grevet, 1999; Shu et al., 1999).

A via dopaminérgica parte da substância negra e da área tegmental ventral para o estriado (núcleos caudado e putâmen) e para as regiões mesolímbico/mesocorticais, respectivamente (Figura 2). As vias noradrenérgica, colinérgica e a via serotoninérgica estão anatomicamente muito próximas e isto parece estar associado a várias funções regulatórias do Sistema Nervoso Central, tais como o controle motor, a memória, o humor, a motivação e o estado metabólico, nas quais a dopamina, a noradrenalina, a acetilcolina e a serotonina estão implicados (Cases et al., 2000; Mongeau et al., 1997; Stiedl et al., 2000).

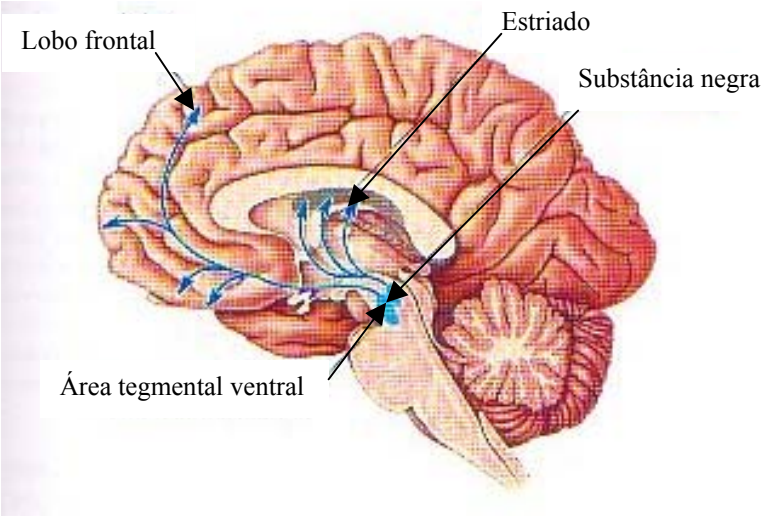

Figura 2. O sistema dopaminérgico e suas conexões (Adaptado de Bear et al., 2002)

A APO, sendo um agonista dopaminérgico, é freqüentemente utilizada em estudos do comportamento associados ao sistema dopaminérgico (Davies et al., 1974; Ichihara et al., 1988; Dalley et al., 2002; Estrella et al., 2002; Ellenbroek e Cools, 2002). O comportamento estereotipado induzido pela APO já está bem estabelecido. Recentemente, dois dos diferentes comportamentos estereotipados induzidos pela APO descritos em ratos, um caracterizado pela ação repetitiva do animal de farejar e o outro de lamber e/ou roer, estão sendo atribuídos ao polimorfismo dos receptores e transportadores dopaminérgicos, além de fatores ambientais (Germeyer et al., 2002). Outros estudos mostram uma dissociação entre o comportamento estereotipado espontâneo e o induzido pela APO em roedores, sendo que o primeiro parece ser mediado por sub-tipos de receptores dopaminérgicos ou por um mecanismo independente de DA (Powell et al., 1999; Battisti et al., 2000c; Presti et al., 2002).

A sensibilização do comportamento estereotipado ocorre quando há uma intensificação deste comportamento devido à administração de uma dose alta ou de doses repetidas de uma droga psico-estimulante. Este aumento da resposta parece estar relacionado a alterações na via dopaminérgica, possivelmente associadas à ativação do receptor glutamatérgico N-metil-D-aspartato (NMDA), no desenvolvimento da sensibilização. Antagonistas do receptor NMDA parecem modular indiretamente a transmissão dopaminérgica e bloquear a expressão de sensibilização dependente de contexto (ambiente) induzida por APO e anfetamina (Battisti et al., 2000a; Battisti et al., 2000b; Peeters et al., 2002).

Em estudos para a avaliação de seus efeitos cognitivos, APO (0.5 mg/Kg) inibiu a memória de uma tarefa de esquiva inibitória em ratos, com efeito amnésico quando administrada durante o treino (0 h), e no pós-treino (10-12 h). Os efeitos inibitórios sobre a aquisição e a consolidação da memória foram atribuídos à ligação da APO a receptores D1, uma vez que os efeitos foram revertidos pelo co-tratamento de um antagonista seletivo D1, SCH 23390 ((R)-(+) –8-cloro –2,3,4,5-tetraidro-3-metil-5-fenil-1H-3-benzazepin-7-ol), mas não por sulpirida, um antagonista específico D2. APO não alterou o comportamento dos animais no campo aberto imediatamente após a administração e 24 h mais tarde (Doyle e Regan, 1993; Doyle et al., 1996).

2. ESPÉCIES REATIVAS DE OXIGÊNIO (ERO)

Os radicais livres são definidos como átomos ou moléculas com um ou mais elétrons desemparelhados em seu orbital mais externo. O termo ERO – espécies reativas do oxigênio – abrange coletivamente os radicais de oxigênio ($O_2^{\bullet -}$, radical superóxido; $OH^{\bullet}$, radical hidroxil; $RO_2^{\bullet}$, radical peroxil; $RO^{\bullet}$, radical alcoxil) e os derivados não

radicais potencialmente oxidantes (por exemplo o H_2O_2, peróxido de hidrogênio) (Engelhardt, 1999; Halliwell e Gutteridge, 2000). As ERO são produzidas normalmente nos seres vivos como conseqüência de diversos processos metabólicos, que envolvem reações de transferência de elétrons. Durante o metabolismo respiratório, a maior parte do oxigênio consumido é reduzido na mitocôndria a água pela cadeia respiratória, sendo que ATP (trifosfato de adenosina) é sintetizado pela fosforilação oxidativa. Entretanto, cerca de 2 a 5% do oxigênio sofre redução incompleta, produzindo espécies parcialmente reduzidas tais como o radical superóxido $O_2^{\bullet-}$, o peróxido de hidrogênio H_2O_2, e o radical hidroxila $OH^{\bullet}$ altamente reativo, as quais são resultantes respectivamente da redução por um, dois e três elétrons (Figura 3) (Boveris, 1998; Halliwell e Gutteridge, 2000).

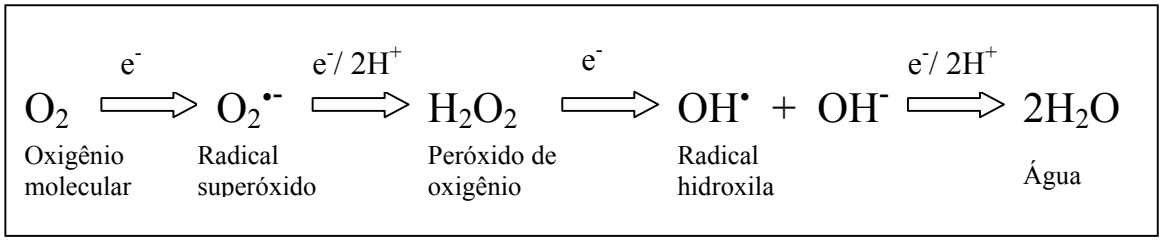

Figura 3. Redução univalente da molécula de oxigênio (Adaptado de Boveris, 1998)

Os metais de transição, tais como ferro e cobre (Fe^{2+} e Cu^{+}), aumentam a velocidade da produção de $OH^{\bullet}$ (revisado em Linder, 2001; De Freitas e Meneghini, 2001). A reação envolvendo metais ocorre em etapas, onde primeiramente os íons férrico (Fe^{3+}) ou cúprico (Cu^{2+}) são reduzidos pelo radical superóxido, produzindo íons ferroso (Fe^{2+}) ou cuproso (Cu^{+}) e oxigênio molecular (Reação de Haber-Weiss) (Figura 4). Estes íons reagem rapidamente com H_2O_2, gerando, desta forma, radical hidroxila ($OH^{\bullet}$), íon hidroxila (OH^{-}) e Fe^{3+} (Reação de Fenton) (Figura 4). Por esta razão, as

células mantém um rígido controle da homeostase metálica. O transporte de metais é altamente regulado e os íons de metais de transição são mantidos em sua valência mais alta ou estão de alguma forma complexados a enzimas e proteínas onde são armazenados ou fazem parte funcional das mesmas. A reação de redução de Fe^{+3} pelo $O_2^{\bullet-}$ acontece *in vitro*, porém *in vivo* parece não ocorrer desta forma por praticamente não haver íons de ferro livres. *In vivo* o radical $O_2^{\bullet-}$ oxida centros metálicos [4Fe-4S] de desidratases, como a aconitase, liberando o Fe^{+2} que então serve de catalisador para a formação do radical $OH^{\bullet}$ a partir do H_2O_2 (Thomas et al., 1998).

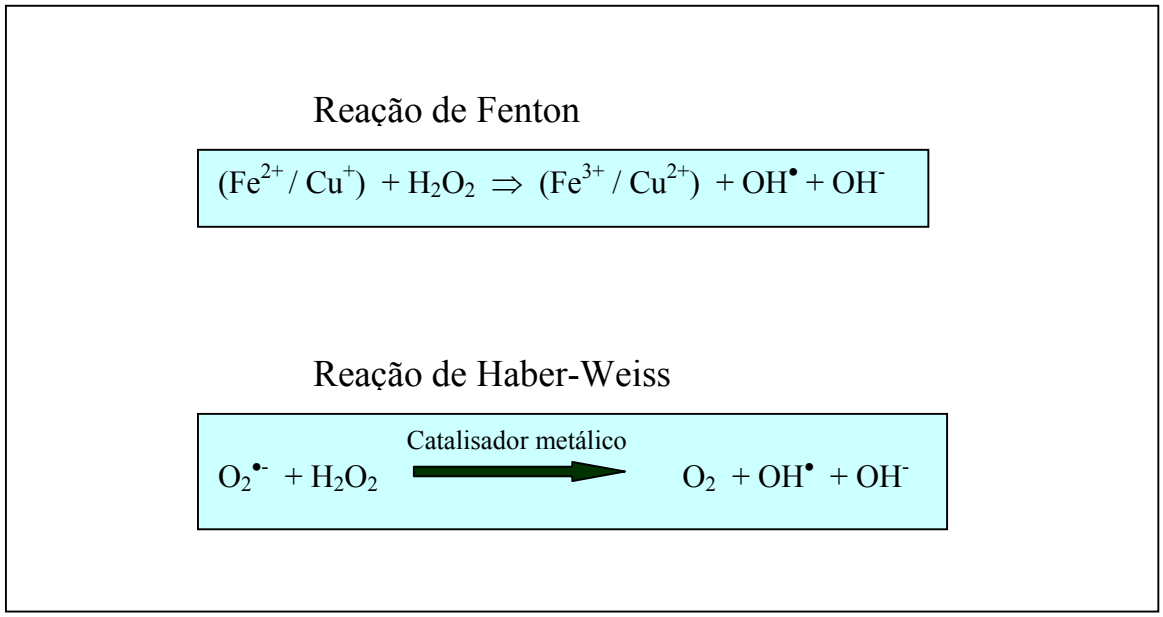

Figura 4. Representação da formação do radical $OH^{\bullet}$ pelas reações de Haber-Weiss / Fenton.

O superóxido que é gerado como um sub-produto do metabolismo normal, particularmente na mitocôndria, também é formado por enzimas específicas como NADPH oxidase e xantina oxidase, e nos tecidos como o fígado, envolvido no

metabolismo de xenobióticos (Boveris, 1998; Thomas et al., 1998; Srinivasan et al., 2000). Acredita-se que a membrana da mitocôndria, considerada a maior fonte de $O_2^{\bullet-}$ é muito pouco permeável ao mesmo. Do contrário, o H_2O_2 por ser uma molécula neutra pode atravessar as membranas com relativa facilidade, e atingir alvos distantes do local de sua formação. O H_2O_2 pode ser gerado principalmente na reação de dismutação do $O_2^{\bullet-}$ que ocorre de forma lenta espontaneamente mas aumenta pelas enzimas superóxido dismutase (SOD) e nos fagócitos. O H_2O_2 também é um subproduto da assimilação oxidativa de várias fontes de carbono e de nitrogênio por peroxissomos e glioxissomos (Fridovich, 1998). Os efeitos mais tóxicos provocados pela formação tanto de $O_2^{\bullet-}$ quando de H_2O_2 é a subsequente e rápida formação do radical hidroxil ($OH^{\bullet}$), via reação de Fenton (Figura 4). O radical $OH^{\bullet}$ tem uma meia-vida curtíssima e sua difusão é limitada pela sua velocidade de reação. Por isto, a melhor defesa que a célula tem contra este radical é preventiva (Chaudière e Ferreri-Iliou, 1999; Halliwell e Gutteridge, 2000).

Há um grande número de compostos químicos supostamente ativados pelo oxigênio via ciclo redox, entre os quais os que apresentam grupamentos catecólicos e quinônicos. O passo inicial para a autoxidação de catecóis e catecolaminas e outras hidroquinonas (QH) envolve a transferência de elétrons do grupamento catecólico ou hidroquinônico para o oxigênio molecular (equação 1), sendo que esta transferência pode ser mediada por metais de transição (Kalyanaraman et al., 1985; Miller et al., 1990). O radical superóxido formado nesta etapa inicial da autoxidação de catecolaminas, também pode reagir novamente com a forma reduzida deste composto, gerando ERO (equação 2) (Kalyanaraman, 1990; Smythies, 1997; Dunnett e Björklund, 1999).

$$QH + O_2 \xrightarrow{-2H^+} QH^{\bullet} + O_2^{\bullet -} \quad \text{(equação 1)}$$

$$QH + O_2^{\bullet -} \rightarrow QH^{\bullet} + H_2O_2 \quad \text{(equação 2)}$$

2.1 Danos oxidativos ao DNA

As ERO são capazes de danificar diferentes biomoléculas, tais como lipídios, proteínas, glicídios e ácidos nucleicos (RNA, DNA), comprometendo, desta forma, a integridade celular. No DNA, as ERO produzem uma série de lesões, danificando bases, deoxiriboses, causando quebras simples de cadeia, criando sítios apurínicos apirimidínicos (sítios AP) e ligações cruzadas entre DNA e proteínas (Wang et al., 1998; Laval et al., 1998). Sabe-se que nem o radical superóxido nem o H_2O_2 atacam o DNA diretamente, porém o radical hidroxila e também o oxigênio singlet podem provocar lesões diretas ao DNA (Cadet et al., 1999; Meehan et al., 1999; Brozmanová et al., 2001). As ERO produzem mais de 30 diferentes adutos de base. Uma das mais importantes modificações de bases geradas pelo radical hidroxila é a 7,8-diidro-8-oxo-2'-deoxiguanina (8-oxo-G), devido à oxidação do átomo C8 do anel da guanina. O 8-oxo-G é o produto de dano oxidativo de base melhor caracterizado, sendo fortemente mutagênico *in vitro* e *in vivo*, causando preferencialmente transversões G→T, quando não reparado (Wang et al., 1998; Brozmanová et al., 2001). O ataque a purinas provoca também a formação de formamidopirimidinas (Fapy), produtos da abertura do anel imidazólico. Em relação às pirimidinas, o radical hidroxila reage preferencialmente com ligações duplas C5-C6, formando glicóis de timina e citosina. A base oxidada 5'hidroxi-2'-deoxicitidina, causa transições C→T, e a timina glicol causa transições T→C, sendo altamente mutagênicas. As riboses são alvos menos freqüentes, porém o ataque do

radical hidroxila pode abstrair um dos átomos de hidrogênio da ribose, formando radicais que podem levar a quebras simples de cadeia, sítios abásicos e sítios álcali-lábeis (revisado em Brozmanová et al., 2001).

2.2 Defesas antioxidantes

Para a manutenção da integridade do DNA e de todos os componentes celulares, os organismos, tanto procariotos quanto eucariotos, apresentam normalmente sistemas de defesa que incluem três níveis de proteção: (a) prevenção da formação de ERO, (b) intercepção de ERO geradas e (c) reparação de danos ocasionados pelas ERO (Demple e Harrison, 1994; Sies, 1997; Henriques et al., 2001). Além destes, há mecanismos de regulação da capacidade antioxidante que modulam a manutenção e a adaptação de níveis adequados de defesas contra ERO (Gonzàlez-Flecha e Demple, 1999; Delaunay et al., 2000). A proteção antioxidante pode ser enzimática e não-enzimática. Halliwell e Gutteridge (2000) definem como antioxidante "qualquer substância que, quando presente em baixas concentrações, comparadas às de um substrato oxidável, retarda ou previne significativamente a oxidação deste substrato". As defesas antioxidantes enzimáticas são representadas principalmente pelas enzimas superóxido dismutase (SOD), catalase (CAT) e peroxidase (glutationa peroxidase, Gpx) (Figura 5) e as não-enzimáticos pelas moléculas que podem agir como seqüestradoras de radicais ("scavenger" ou "chain-breaking") ou da energia de excitabilidade de moléculas ("quencher") e quelante de metais de transição (revisado em Henriques et al., 2001). A produção excessiva de ERO ou a diminuição das defesas antioxidantes, ou seja, o desbalanço entre pró-oxidantes e antioxidantes, gera um desequilíbrio no estado redox celular conhecido como estresse oxidativo (Henriques et al., 2001). O estresse oxidativo

pode estar relacionado com vários processos tais como, mutagênese, carcinogênese, doenças degenerativas e alterações do envelhecimento (Engelhardt, 1999; Halliwell e Gutteridge, 2000).

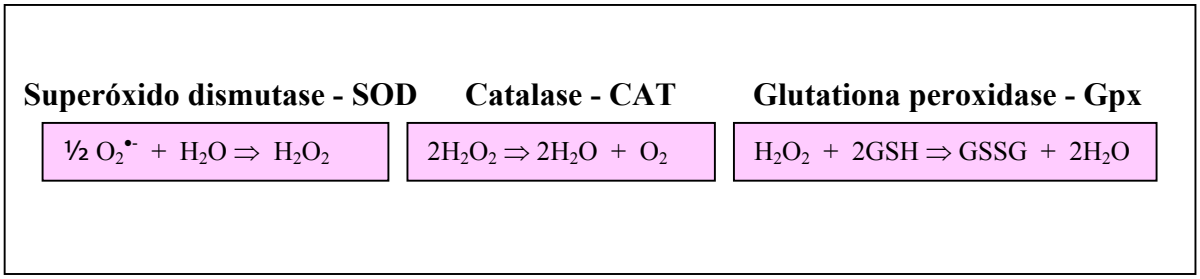

Figura 5. Detoxificação de ERO pelas enzimas SOD, CAT e Gpx

O controle primário da resposta ao estresse oxidativo pelos organismos encontra-se na transcrição de genes responsáveis pela síntese de proteínas envolvidas na proteção celular (Zheng et al., 1998; Gonzàlez-Flecha e Demple, 1999; Lee et al., 1999; Engelhardt, 1999; Cyert, 2001). Muito do conhecimento adquirido sobre a resposta transcricional foi obtido através de linhagens bacterianas, principalmente *Escherichia coli* e *Salmonella typhimurium* (Demple, 1991; Zheng et al., 1998; Ruiz-Laguna et al., 2000). Nestes organismos, o peróxido de hidrogênio induz a ativação de um fator de transcrição, denominado OxyR, o qual promove a transcrição de pelo menos nove genes cujos produtos estão envolvidos na detoxificação de ERO, como por exemplo o gene da catalase-hidroperoxidase I (*katG*), alquil-hidroperoxidase NADPH-dependente (*ahpFC*), glutationa redutase (*gorA*) (Ruiz-Laguna et al., 2000). A expressão daqueles genes ocorre rapidamente em resposta a concentrações micromolares de H_2O_2 adicionadas ao meio de cultura (Zheng et al., 1998). Além disso, estas bactérias apresentam uma resposta adicional, induzida pela presença de radical superóxido, que é

regulada pelos produtos gênicos de SoxR e SoxS, que atuam na expressão dos genes da superóxido dismutase (*sodA*), glicose-6-fosfato desidrogenase (*zwf*), endonuclease IV (*nfo*), entre outros (Hidalgo et al., 1998).

Em eucariotos, o estado redox intracelular é um componente crítico nas respostas ao estresse oxidativo, e responde especialmente ao equilíbrio tiol-dissulfeto. Sistemas endógenos da glutationa e tiorredoxina, portanto, podem ser considerados moduladores efetivos da expressão gênica sensível à regulação redox (Sen e Packer, 1996; Izawa et al., 1999; Nguyên et al., 2001; Kuge et al., 2001). Uma cascata de sinais segue até a modulação gênica em resposta ao estado redox da célula. Alguns fatores de transcrição são diretamente regulados pela homeostase redox. Ref-1 ("redox factor 1") é uma proteína nuclear que medeia a regulação redox de proteínas nucleares, inclusive NF-κB, AP-1 e p53, através de uma ação da tioredoxina sobre resíduos de cisteína. Sítios de ligação do fator de transcrição NF-κB são encontrados em promotores de uma série de genes principalmente relacionados com respostas inflamatórias tais como as citoquinas citotóxicas (TNF-α, IL-1 e IL-6) (Engelhardt, 1999; Cyert, 2001). A ativação dos genes regulados por NF-κB está envolvida diretamente com diversas patologias, incluindo a Doença de Parkinson (Youdim et al., 1999).

3 AVALIAÇÃO DA ATIVIDADE GENOTÓXICA

Os testes de genotoxicidade detectam efeitos de substâncias tóxicas para o genoma. Os mais utilizados são aqueles que detectam mutações em células germinativas ou somáticas, por exemplo mutação gênica, associada a alterações na seqüência de nucleotídeos do DNA, ou ao nível cromossômico, como aberrações e micronúcleos (Waters et al., 1999; MacGregor et al., 2000; Dearfield et al., 2002). Uma vez lesado

seu DNA, as células respondem utilizando diferentes estratégias de ação, tais como morte por citotoxicidade ou apoptose, modulação da expressão gênica controlando o ciclo celular, e reparação do material genético por via livre ou sujeita a erro, sendo a segunda responsável pela fixação das mutações. Normalmente é a combinação destes fatores que compõem a resposta a danos genéticos (Friedberg et al., 1995; Moustacchi, 2000).

Os métodos que são mais amplamente empregados para a detecção de mutações gênicas são aqueles que utilizam as bactérias (*Salmonella typhimurium* e *Escherichia coli*) (Waters et al., 1999; MacGregor et al., 2000; Dearfield et al., 2002). Estes são relativamente simples e reprodutíveis. Para se obter dados sobre mutações gênicas em eucariotos empregam-se testes em leveduras (*Saccharomyces cerevisiae*), em células de mamíferos, em *Drosophila*, ou mesmo mutações somáticas em mamíferos pelo teste de HGPRT (gene de hipoxantina-guanina fosforibosil-transferase, ligado ao cromossomo X de mamíferos), ou o "mouse spot test" (alteração da cor do pelo em camundongos tratados durante a embriogênese). Para a detecção de mutações cromossômicas, os testes mais utilizados incluem as aberrações cromossômicas e micronúcleos, sendo que estes testes exigem que a célula esteja em estado proliferativo (Tice et al., 1988; MacGregor et al., 2000). Estes testes têm um sistema de validação internacional, e podem ser desenvolvidos tanto *in vitro* quanto *in vivo*. Recentemente o teste alcalino eletroforético de célula única ou Ensaio Cometa (EC), que detecta quebras no DNA, também tem sido recomendado (Fairbairn et al., 1995; Singh e Stephens 1996; Singh, 2000), sendo este realizado em células individuais não necessariamente em proliferação.

3.1 Teste *Salmonella/*microssoma

O teste *Salmonella*/microssoma, desenvolvido pelo Dr. Bruce Ames e colaboradores na década de 70 (Ames et al., 1975; Ames, 1979) é um ensaio bacteriano de curta duração que busca identificar substâncias que causem danos genéticos e possam evoluir a mutações (para revisão, ver Maron e Ames, 1983 e Mortelmans e Zeiger, 2000). Este ensaio baseia-se na indução de mutações reversas em linhagens de *Salmonella typhimurium* auxotróficas para o aminoácido histidina que conduzem as mesmas à prototrofia.

Em bactérias, danos ao DNA ou inibição na replicação podem desencadear uma série de alterações fenotípicas denominadas de Funções SOS. Esta resposta depende da expressão dos alelos selvagens de recA e lexA e envolve a expressão de pelo menos 27 genes, muitos dos quais envolvidos em processos de reparação do DNA (Figura 6). Na forma desligada do sistema, a proteína LexA está reprimindo os genes alvos. Quando ocorre um dano ao DNA é emitido um sinal indutor que ativa RecA na presença de ATP. Esta forma ativada da protease RecA induz uma clivagem proteolítica do repressor LexA, em uma ponte Ala-Gly, que resulta na desrepressão do "regulon", possibilitando a expressão ou superexpressão dos genes alvo controlados pelas funções SOS. Conforme a lesão é reparada o sistema volta a sua forma desligada (revisado em Konola et al., 2000; Keller et al., 2001) (Figura 6).

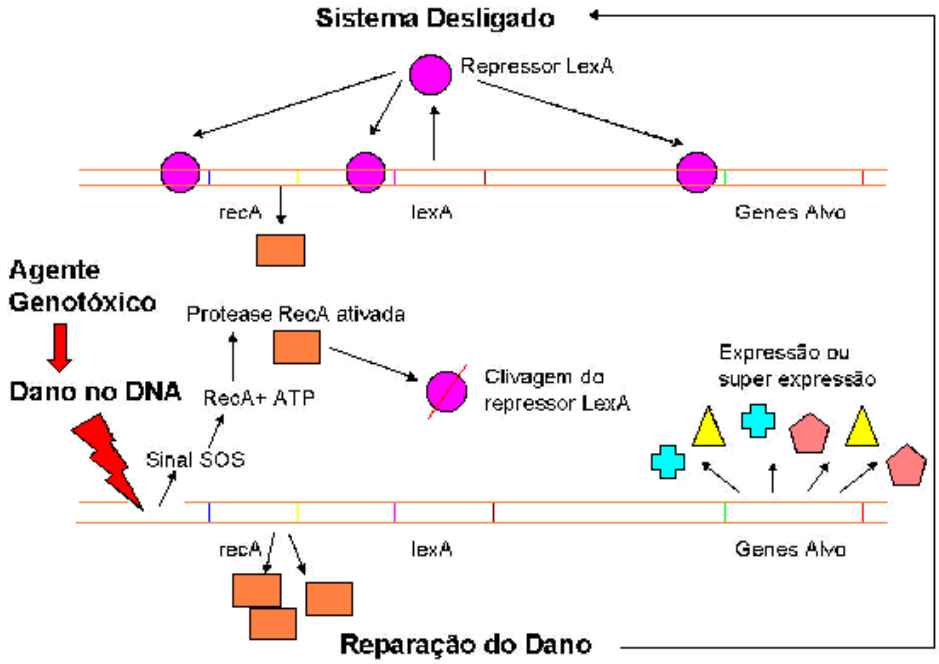

Figura 6. Esquema de funcionamento do sistema regulatório SOS (Adaptado de Leitão e Alcantara Gomes, 1997)

Nas linhagens de *Salmonella typhimurium* utilizadas no teste *Salmonella*/microssoma, a resposta SOS é otimizada pela presença do plasmídeo pKM101, carregando os genes mucAB, alelos de umuCD de bactérias, envolvidos na reparação sujeita a erro. Ao se transformar em protease RecA, além de desreprimir o sistema SOS também cliva UmuD, gerando uma forma ativada, UmuD', que se complexa com UmuC. Este complexo se liga à sub unidade ε da DNA polimerase I, permitindo que esta realize a síntese translesão, levando portanto à mutagênese (Leitão e Alcantara Gomes, 1997; Bhamre et al., 2001).

Estas linhagens ainda contam com outras mutações que também auxiliam a identificação de substâncias que lesam o DNA: mutação no gene *rfa*, que acarreta na perda parcial da barreira de polissacarídeos da parede bacteriana, aumentando a permeabilidade e facilitando a entrada de moléculas maiores na célula; deleção do gene

uvrA, responsável pelo sistema de reparação por excisão de nucleotídeos (NER) do DNA, o que impede que esta via de reparação esteja atuante nestas linhagens, possibilitando a fixação das mutações (Maron e Ames, 1983; Mortelmans e Zeiger, 2000).

Cada linhagem é mutada de forma diferente no operon da histidina, o que permite que tenham especificidade na detecção de um determinado tipo de mutágeno:

A linhagem TA98 detecta mutágenos que causam erro no quadro de leitura do DNA ("frameshift mutation") apresentando como ponto preferencial oito resíduos repetitivos GC $\begin{matrix}\text{-CGCGCGCG-}\\ \text{-GCGCGCGC-}\end{matrix}$ no operon do gene his_{D3052} que codifica para enzima histidinol desidrogenase (Levin et al., 1982a; Maron e Ames, 1983; Mortelmans e Zeiger, 2000).

A linhagem TA100 detecta mutágenos que causam substituição de pares de base do DNA, através de uma mutação no gene his_{G46} que codifica a primeira enzima da biossíntese da histidina. Tem como ponto preferencial para a reversão o par GC, sendo a mutação a substituição de uma prolina $\begin{matrix}\text{-GGG-}\\ \text{-CCC-}\end{matrix}$ por uma leucina $\begin{matrix}\text{-GAG-}\\ \text{-CTC-}\end{matrix}$ (Barnes et al, 1982; Maron e Ames, 1983; Mortelmans e Zeiger, 2000).

A linhagem TA97 apresenta uma seqüência de seis citosinas no sítio de mutação no gene his_{D6610} . A especificidade mutagênica desta linhagem é similar à da linhagem TA1537 mas sua sensibilidade é muito maior. A TA97 apresenta ainda uma seqüência de pares de bases G-C alternadas próxima à seqüência de citosinas, semelhante ao sítio da mutação his_{D3052} nas linhagens TA98 e TA1538. Estas seqüências de bases repetitivas representam "hot-spots" para mutágenos que causam erro no quadro de leitura ("frameshift mutation") (Levin et al., 1982a; Mortelmans e Zeiger, 2000).

A linhagem TA102, diferindo das outras linhagens descritas, contêm pares de bases AT no sítio mutante his_{G428}. Esta mutação foi introduzida no plasmídio multicópia pAQ1, com objetivo de amplificar o número de sítios alvo. A mutação his_{G428} é uma mutação *ochre* (TAA) no gene *hisG*, a qual pode ser revertida por todas as seis possibilidades de trocas de pares de bases (transversão/transcrição). Esta mutação também é revertida por agentes que causam danos oxidativos. Como o gene *uvrB* não foi deletado, a bactéria é proficiente no sistema de reparação NER, aumentando assim a sua habilidade para detectar agentes indutores de pontes entre as cadeias do DNA ("interstrand DNA cross-linking") (Levin et al., 1982b; Maron e Ames, 1983; Mortelmans e Zeiger, 2000).

Em testes com procariotos um componente importante é o sistema de indução microssomal. Este sistema enzimático é extraído de células de fígado de ratos pré-tratados com um indutor enzimático e tem a função de reproduzir o metabolismo de mamíferos, permitindo que também seja avaliada a ação genotóxica dos metabólitos dos agentes testados. A função primária deste sistema microssomal é proteger a célula, degradando fisiologicamente substâncias inerentes ao metabolismo celular e desativando substâncias estranhas à célula. Porém, algumas substâncias (pró-mutágenos) inativas quando em contato direto com a célula, podem gerar metabólitos eletrofílicos que reagem com os centros nucleofílicos do DNA formando adutos (Crosby, 1998; Mortelmans e Zeiger, 2000).

A combinação de testes com presença e ausência de sistema metabólico e linhagens bacterianas sensíveis a diferentes agentes possibilita determinar tanto substâncias com ação genotóxica direta como monitorar a atividade, positiva ou negativa, dos metabólitos gerados pela biotransformação.

3.2 Testes em linhagens WP2 de *Escherichia coli*

O ensaio de mutação reversa ao triptofano em linhagens da bactéria *Escherichia coli* é utilizado em conjunto com o teste *Salmonella*/microssoma para identificar substâncias com atividade genotóxica. As linhagens de WP2 *E. coli* apresentam em comum um par de base AT, no sítio crítico de mutação dentro do gene *trpE65*, que bloqueia a biossíntese do triptofano, num passo anterior ao da formação do ácido antranílico (Ohta et al., 1998; Watanabe et al., 1998a, 1998b). A OECD ("Organinisation for Economic Co-operation and Development"), entre outros órgãos internacionais de regulamentação, recomendam estas linhagens em substituição ou em adição a TA102, que também apresenta um par AT no sítio de mutação. Green e Muriel (1976) publicaram, pela primeira vez, os procedimentos padrões para o teste de reversão em WP2 de *E. coli* que poderiam ser utilizados na rotina, para a identificação de substâncias genotóxicas. Eles recomendaram o uso de linhagens proficiente e deficiente no reparo do DNA, contendo a mutação no gene *trpE*. As primeiras linhagens WP2/pKM101 foram construídas por Monti-Bragadin e colaboradores (1976), pela introdução do plasmídio pKM101 em linhagens de WP2 com diferentes habilidades de reparo do DNA. Atualmente, as linhagens WP2 com a mutação *trpE* mais utilizadas são as WP2 (tipo selvagem, proficiente no reparo do DNA), WP2/pKM101, WP2 *uvrA* e WP2 *uvrA* /pKM101 e os procedimentos metodológicos são similares aos utilizados no teste *Salmonella*/microssoma (Mortelmans e Riccio, 2000).

Para melhor detectar substâncias que induzem mutações por danos oxidativos, Blanco e colaboradores (1998) desenvolveram a linhagem IC203, derivada de WP2 *uvrA*/pKM101, deficiente na função OxyR. A deficiência em OxyR bloqueia a síntese de enzimas antioxidantes, tais como catalase-peroxidase, alquil hidroperoxidase e

glutationa redutase, aumentando a sensibilidade da bactéria à mutagênese resultante de lesões ao DNA causada por ERO. O teste de mutagenicidade realizado com as linhagens IC188 (WP2 *uvrA*/pKM101) e IC203 (WP2 *uvrA oxyR*/pKM101) é chamado de Mutoxiteste-WP2 (Blanco et al., 1998). Num dos estudos de validação deste teste, foram avaliados 80 substâncias, principalmente agentes oxidantes conhecidos, de diversas classes e estruturas, tais como peróxidos, quinonas, derivados fenólicos e catecolaminas, sendo que mais de 50 delas mostraram resultados positivos para indução de mutações por danos oxidativos (Martínez et al., 2000).

3.3 Ensaio Cometa

O Ensaio Cometa (EC) (ou "SCGE – single cell gel electrophoresis") é uma técnica rápida e sensível para mensurar e analisar lesões e detectar efeitos de reparo no DNA (Singh et al., 1988; Fairbairn et al., 1995; Liu et al., 2001) de células expostas à agentes genotóxicos. Este teste apresenta algumas vantagens sobre os testes bioquímicos e citogenéticos, como: (a) versatilidade em relação ao tipo de célula a ser analisada, sendo que o método já foi adaptado a qualquer tipo de célula, além de ser aplicado a diversos organismos; (b) necessidade de somente um pequeno número de células; (c) não ser necessário células em divisão. Pode ser usado em testes *in vitro* e *in vivo* de indução de danos ao DNA (Collins et al., 1997; Tice et al., 2000; Sekihashi et al., 2002; Valverde et al., 2002). Os danos que são mais facilmente mensurados pelo teste são quebras no DNA (simples e duplas), danos álcali-lábeis, ligações cruzadas e quebras resultantes de reparo por excisão não concluídas (Singh et al., 1988; Fairbairn et al., 1995; Hartmann e Speit, 1997, Tice et al., 2000). Em testes utilizando animais, é possível avaliar o potencial genotóxico de uma substância em células de órgãos e/ou

tecidos alvo específicos (Sasaki et al., 1998; 1999; 2002), como por exemplo a atividade genotóxica de uma neurotoxina no cérebro (Shen et al., 2001; Morris et al., 1999; Martin e Liu, 2002).

Existem basicamente dois tipos de protocolos para este teste: (a) tratamento neutro, que detecta quebra dupla no DNA; e (b) tratamento alcalino, que detecta quebras simples de cadeia, diretas ou induzidas por lesões nas bases do DNA (como oxidação, metilação o outros adutos), cujo procedimento transforma essas anomalias em lesões álcali-lábeis (Fairbairn et al., 1995). Esta técnica tem sido modificada também para detectar adutos no DNA (dímeros de timina, danos oxidativos), usando anticorpos específicos (Sauvaigo et al., 1998) ou enzimas de reparo do DNA (Collins et al., 1995; Speit e Hartmann, 1999).

A técnica consiste em submeter as células, embebidas em gel de agarose sobre uma lâmina, a uma eletroforese que faz migrar pela corrente elétrica os segmentos de DNA livres, resultantes de quebras, para fora do núcleo. Após a eletroforese, as células que apresentam um núcleo redondo são identificadas como normais, sem dano reconhecível ao DNA. As células lesadas são identificadas visualmente por uma espécie de cauda, como de um cometa, formada pelos fragmentos de DNA (Figura 7). Estes fragmentos podem se apresentar em diferentes tamanhos e ainda estar associados ao núcleo por uma cadeia simples (Fairbairn et al., 1995).

A identificação do dano pode ser feita por diferentes maneiras, uma forma é medir o comprimento do DNA migrante com a ajuda de uma ocular micrométrica, outra é classificar visualmente as células com dano em diferentes classes (0-4), podendo obter um valor arbitrário, que expresse o dano geral que uma população de células sofreu (Collins et al., 1997; Silva et al., 2000; Boeira et al., 2001). O crescente interesse e

importância do Ensaio Cometa levou o desenvolvimento de um analisador de imagens, que serve para quantificar a cromatina que migrou para fora do núcleo.

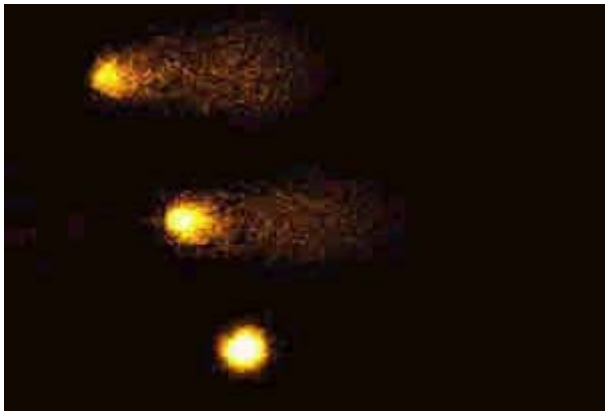

Figura 7. Visualização da imagem de células "cometa", onde a cabeça representa o núcleo original e a "cauda" os fragmentos de DNA

4. AVALIAÇÃO DA ATIVIDADE ANTIGENOTÓXICA

Alterações metodológicas dos testes de genotoxicidade podem ser empregadas para identificar compostos que estariam envolvidos na proteção do DNA, através de uma atividade antigenotóxica (Lohman et al., 2001; Weisburger, 2001). Esta é uma das atividades mais importantes para a determinação de agentes quimiopreventivos, naturais ou sintéticos, envolvidos na modulação ou inibição de processos patológicos, resultantes da exposição a genotoxinas e agentes carcinogênicos presentes no ambiente (De Flora 1988; De Flora e Ramel, 1988; Heo et al., 2001; Ames, 2001, Morrow et al., 2001). Muitos estudos indicam que algumas plantas medicinais e os alimentos de origem vegetal são uma excelente fonte de agentes quimiopreventivos (Mitscher et al., 1992; Lee et al., 1998; Cabrera, 2000; Ames, 2001). Entre os possíveis quimiopreventivos destacam-se as vitaminas (como por exemplo, as vitaminas E e C) e

os polifenóis (Odin, 1997; Ferguson, 2001). Porém, a natureza química de muitos outros compostos e os mecanismos de proteção ainda permanecem pouco conhecidos (Duthie et al., 1997; Dhawan et al., 2002).

Agentes inibidores de mutagênese ou antimutagênicos têm sido avaliados pelo uso de ensaios rápidos, particularmente o teste *Salmonella*/microssoma, que auxilia na elucidação do mecanismo antimutagênico (Minnunni et al., 1992; Edenharder et al., 1999; Karekar et al., 2000). De Flora e colaboradores (1998; 2001) propuseram uma classificação detalhada de possíveis mecanismos de modulação extra- e intracelulares que podem prevenir mutações e câncer, incluindo a inibição de efeitos genotóxicos, atividade antioxidante, inibição da proliferação celular, indução de diferenciação celular e interferência com vias de transdução de sinais. O bloqueio de espécies químicas eletrofílicas representa um dos mais promissores mecanismos que levam à atividade antimutagênica e anticarcinogênica. A combinação de eletrófilos mutagênicos com centros nucleofílicos do DNA pode ser bloqueada pelos inibidores de mutagenicidade principalmente (a) pela sua interação com alvos do DNA protegendo-os; (b) reação direta com o mutágeno, provocando inativação; (c) inibição de enzimas de fase I envolvidas na ativação de pró-mutágenos, como certas monoxigenases citocromo P-450-dependentes, e/ou (d) indução de enzimas de fase II, responsáveis pela desativação do mutágeno ou de seus intermediários reativos, tais como as GSH-S-transferases, glucuronosil transferases e sulfotransferases (De Flora et al., 2001; Kong et al., 2001). Os mecanismos da antimutagenicidade normalmente são complexos, e podem depender do sistema biológico utilizado e do tipo de mutágeno. Além disso, muitos agentes químicos que inibem a mutagenicidade sob certas condições experimentais podem se

tornar ineficazes ou até mesmo deletérios quando em outras condições (Lohman et al., 2001; De Flora et al., 2001).

A maioria dos testes de mutagenicidade em *S. typhimurium* e *E. coli* detecta agentes que induzem mutações reversas como resultado da formação de adutos ou por intercalação entre pares de bases do DNA (Waters et al., 1999; Rosenkranz, 2002; Dearfield et al., 2002). Estudos mais recentes têm revelado que muitas mutações são também induzidas como conseqüência de danos oxidativos ao DNA, e estes seriam a maior causa de câncer em humanos, especialmente por substâncias endógenas (Laval et al., 1998; Rosenkranz, 2002). Por isso há um grande interesse na busca de novos agentes que possam proteger o genoma por mecanismos antioxidantes (Ferguson, 2001; Shukla et al., 2002).

Os testes *Salmonella*/microssoma e Mutoxiteste-WP2 podem detectar agentes que inibem a atividade mutagênica induzida por mutágenos oxidantes ou geradores de ERO, como terc-butil-hidroperóxido (t-BOOH) e H_2O_2. Tais agentes têm sido considerados antimutágenos por um mecanismo antioxidante, devido à proteção que conferem ao DNA contra danos oxidativos (Minnunni et al., 1992; Edenharder et al., 1999; Chaudiére e Ferrari-Iliou, 1999; Kaur e Saini, 2000; Karekar et al., 2000). Em alguns estudos, a vitamina E apresentou atividade antimutagênica contra mutágenos oxidantes, devido às suas propriedades "scavenger" (revisado em Claycombe e Meydani, 2001).

5. A LEVEDURA *SACCHAROMYCES CEREVISIAE* COMO MODELO DE ESTUDO

5.1 Aspectos gerais

A levedura *Saccharomyces cerevisiae* é um organismo eucarioto amplamente estudado, tornando-se ferramenta importante nas pesquisas sobre mutagênese, reparo do DNA e mecanismos que respondem ao estresse oxidativo (Maris et al., 2000; Maris et al., 2001; Boeira et al., 2002; Pungartnik et al., 2002).

A *S. cerevisiae* pertence ao grupo das leveduras anaeróbias facultativas. Isto significa que irá fermentar hexoses como a glicose e a frutose, independente da concentração de oxigênio. A glicose é a fonte de carbono preferida da *S. cerevisiae*, uma preferência que é mediada por um complexo processo de repressão e ativação de genes e proteínas, usualmente conhecido simplesmente como repressão da glicose ou repressão catabólica (revisado em De Winde et al., 1997; Gancedo, 1998). Quando a concentração de glicose cai para menos de 0,2% no meio, há a desrepressão das enzimas que participam da biossíntese de mitocôndrias e de outros genes necessários para o crescimento respiratório (De Winde et al., 1997; Gancedo, 1998). Esse crescimento apresenta fases distintas do ponto de vista metabólico e cinético (Figura 8). Após um breve período de adaptação em meio rico (YPD – 2% glicose), chamado de fase lag, as células iniciam uma divisão celular a cada hora e meia (fase exponencial), com energia proveniente da fermentação da glicose. Ao diminuir a disponibilidade de glicose no meio, ocorre a desrepressão catabólica (transição diáuxica), na qual há uma parada transiente na divisão celular, enquanto as células são preparadas para o metabolismo respiratório. Após, reassume a divisão celular em um ritmo mais lento (uma divisão a cada três ou quatro horas), utilizando o etanol como fonte de carbono, produzido

durante a fermentação (fase pós-diáuxica). Quando todas as fontes de carbono foram exauridas, as células entram na fase estacionária, na qual podem sobreviver por muito tempo na ausência de nutrientes (Pringle e Hartwell, 1982; Fuge e Werner-Washbourne, 1997).

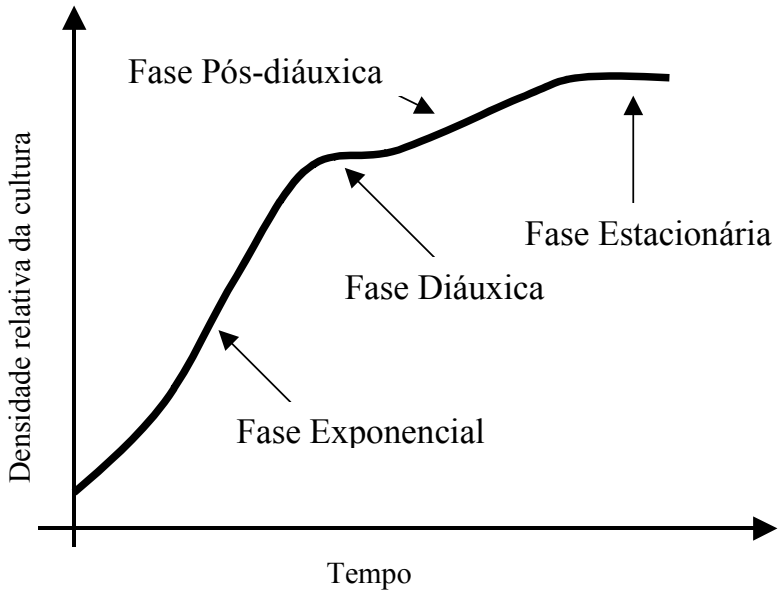

Figura 8. Fases de crescimento da levedura selvagem iniciada em meio completo YPD.

5.2 Defesas antioxidantes da levedura *S. cerevisiae*

Como todos os aeróbios, *Saccharomyces cerevisiae* apresenta uma variedade de mecanismos de defesa contra danos oxidativos via atividades enzimáticas, presença de antioxidantes, seqüestradores de metais e diversos mecanismos de reparação (Maris et al., 2001; Brozmanová et al., 2001; Henriques et al., 2001). Alguns dos produtos de genes envolvidos em estresse oxidativo em *S. cerevisiae* estão na Tabela 1. As superóxido dismutase (SOD) são enzimas que fazem a dismutação do radical superóxido a peróxido de hidrogênio (ver Figura 5). A levedura *S. cerevisiae* contém a

Mn-SOD (produto do gene *SOD2*) localizada na matriz da mitocôndria e CuZn-SOD (produto do gene *SOD1*) presente no citoplasma, núcleo e lisossomos (Gralla e Valentine, 1991; Gralla e Kosman, 1992; Longo et al., 1996; Park et al., 1998; Longo et al., 1999). As linhagens mutante *Δsod1,* que são deficientes nesta enzima, apresentam problemas de crescimento em condições aeróbias, são muito sensíveis à hiperóxia (Bilinski et al., 1985), a substâncias envolvidas em reações tipo ciclo-redox tais como paraquat ou menadiona, perdem a viabilidade em fase estacionária e são auxotróficas para metionina e lisina em presença de oxigênio (Srinivasan et al., 2000). Acredita-se que estas auxotrofias se devem a enzimas relacionadas à síntese destes aminoácidos, que seriam extremamente sensíveis à desativação por $O_2^{\bullet-}$ (Liu et al., 1992, Slekar et al., 1996). Um estudo da super-expressão de uma CuZn-SOD humana em levedura, mostrou que esta enzima promove a resistência das células de levedura contra os agentes estressores que geram ânion superóxido, como paraquat e menadiona e ao choque térmico, mas não contra o H_2O_2 (Yoo et al., 1999). As mutantes *Δsod2* são hipersensíveis ao oxigênio e crescem mal ou não crescem em fontes de carbono não-fermentáveis. A sensibilidade à hiperóxia é revertida pela mutação rho^0 (leveduras sem mitocôndrias), confirmando o papel decisivo da mitocôndria na geração de $O_2^{\bullet-}$ (Guidot et al., 1993; Fernàndez-Checa et al., 1998).

Tabela 1. Produto de alguns genes envolvidos em estresse oxidativo em *S. cerevisiae*

GENE	**PROTEÍNA**	**ATIVIDADE DE DEFESA**
SOD1	Cu/Zn-SOD	Citosólica, inativação do ânion superóxido
SOD2	Mn-SOD	Mitocondrial, inativação do ânion superóxido
CTA1	Catalase A	Peroxisomal, inativação de peróxido de hidrogênio
CTT1	Catalase T	Citosólica, inativação de peróxido de hidrogênio
GSH1	γ-glutamilcisteína sintetase	Primeira enzima da biossíntese de glutationa
GSH2	Glutationa sintetase	Segunda enzima da biossíntese de glutationa
GLR1	Glutationa redutase	Redução de glutationa oxidada
CUP1	Metalotioneína	Quelante de metal
TRX2	Tioredoxina	Agente redutor antioxidante
TRR1	Tioredoxina redutase	Redução de tioredoxina oxidada
YAP1	Fator de transcrição Yap1p	Regula a expressão de genes envolvidos em estresse oxidativo.
SKN7	Fator transcricional Skn7p	Regula a expressão de genes envolvidos no estresse oxidativo
YCF1	Transportador Ycf1p (transportador ABC vacuolar gsh-dependente)	Transportador de metais e drogas para o vacúolo

Adaptado de Maris et al. (2001)

O radical superóxido é mais seletivo em suas reações químicas do que o radical hidroxila que exerce toxicidade devido a danos oxidativos generalizados (Thomas et al., 1998; Halliwell e Gutterigde, 2000; De Freitas e Meneghini, 2001). Um mecanismo proposto da toxicidade do ânion superóxido é baseado na observação de que o superóxido pode especificamente oxidar o sítio [4Fe-4S] de certas enzimas, causando a liberação de ferro do sítio e a inativação da enzima (Srinivasan et al., 2000). Esse processo levaria a um dano oxidativo adicional de outros componentes celulares uma vez que o ferro livre pode promover, via reação de Fenton, a formação do radical hidroxila (Figura 4). A função principal das enzimas SOD seria proteger as proteínas contendo [4Fe-4S], tal como a aconitase mitocondrial (que catalisa a conversão do citrato a isocitrato no ciclo do ácido cítrico), da ação do ânion superóxido, prevenindo o acúmulo de ferro intracelular. Mutantes Δ*sod1* e Δ*sod2* mostraram níveis maiores de ferro, detectado por Ressonância Paramagnética de Elétron (EPR), de até 5 vezes em relação aos níveis basais da linhagem selvagem isogênica e a duplo mutante Δ*sod1*Δ*sod2* apresentou níveis de ferro superiores a 9 vezes o encontrado para a selvagem, em condições aeróbias de crescimento. O tratamento da linhagem selvagem com um gerador de superóxido- paraquat, também aumentou o ferro livre detectável por EPR, indicando que o excesso de ferro livre pode ser devido aos efeitos deletérios do radical superóxido (Srinivasan et al., 2000).

O peróxido de hidrogênio pode ser catabolizado pelas catalases e peroxidases (Tabela 1 e Figura 5). Em levedura, a resistência a peróxido tem sido associada aos níveis intracelulares de glutationa (Izawa et al., 1995; Kobayashi et al., 1996). A glutationa reduzida (GSH) é um tripeptídeo que desempenha função fundamental na proteção das células contra danos oxidativos causado por oxidantes, atuando como

seqüestradora de radicais (Meister, 1995). A utilização de GSH, pela enzima glutationa peroxidase (Gpx), resulta na sua conversão para a forma oxidada (GSSG), a qual é reciclada a sua forma reduzida (GSH) pela glutationa-redutase as expensas de NADPH. O gene *GSH1* (tabela 1) é necessário para a síntese de γ- glutamilcisteína sintetase, a enzima responsável pelo primeiro passo e ponto limitante da biossíntese de GSH, e o gene *GSH2* codifica a glutationa sintetase, a segunda enzima da síntese de GSH. Ambos foram clonados e as mutantes correspondentes analisadas (Ohtake e Yabuuchi, 1991; Lisowsky, 1993; Grant et al., 1997; Brendel et al., 1998; Inoue et al., 1998). Mutantes Δ*gsh1* induzem apoptose (Madeo et al., 1999) e morrem rapidamente quando nenhuma GSH exógena é fornecida. A GSH endógena é importante para a manutenção da integridade mitocondrial; mesmo em meio suplementado com GSH, as mutantes Δ*gsh1* têm uma alta tendência de perder a função mitocondrial (Schmidt et al., 1996; Brendel et al., 1998). Além disso, as leveduras mutantes Δ*gsh1* não adquirem resistência intrínseca sob condições não-fermentáveis e na fase estacionária contra H_2O_2 (Maris et al., 2000) e não mostraram respostas adaptativas ao estresse por H_2O_2 durante a fase exponencial em meio YPD-glicose (Izawa et al., 1995). Duas catalases, uma citosólica e uma peroxisomal, codificadas pelos genes *CTT1* e *CTA1*, respectivamente, também foram identificadas (Spevak et al., 1983; Hartig e Ruis, 1986; Cohen et al., 1988). Ambas são importantes para a resposta adaptativa ao H_2O_2 (Izawa et al., 1996). A regulação do gene *CTT1* pelo H_2O_2 é mediada pelo elemento de resposta ao estresse ("Stress Responsive Elements -STRE"). Leveduras Δ*ctt1* e Δ*cta1* mutantes são bastante sensíveis H_2O_2 (Izawa et al. 1996; Grant et al., 1998).

O fator de transcrição Yap1p é apontado como um dos mais importantes mediadores das respostas adaptativas ao estresse em *S. cerevisiae*, modulando a indução

de genes de defesa contra agentes oxidantes como H_2O_2 (Dormer et al., 2002). Yap1 parece regular genes de forma direta e indireta. Alguns alvos da regulação direta que foram identificados são os genes *TRX2*, um dos dois genes que codificam a tioredoxina em *S. cerevisiae* (Kuge e Jones, 1994; Kuge et al., 2001); *GSH1* (Wu e Moye-Rowley, 1994), *GSH2* (Sugiyama et al., 2000); TRR1, que codifica para tioredoxina redutase (Morgan et al., 1997), *GLR1*, que codifica a glutationa-redutase (Grant et al., 1996a, 1996b), e pelo menos duas bombas, membros da superfamília de transportador envolvidos na resistência múltipla a drogas (MDR): *FLR1*, que codifica o transportador de membrana plasmática Flr1p e *YCF1*, que codifica um transportador (ABC) Ycf1p (bomba de glutationa S-conjugado), presente na membrana vacuolar (Wemmie et al., 1994; Alarco et al., 1997; Bauer et al., 1999). Alguns dos genes regulados por Yap1p requerem a cooperação de outro fator de transcrição, o fator Skn7p (Lee et al., 1999; Dormer et al., 2002).

O Yap1 foi originalmente identificado como um homólogo funcional de AP-1 de mamífero, mostrando habilidade de se ligar ao elemento de reconhecimento de Yap1 (YRE) na região promotora de genes alvo (Izawa et al., 1999; Delaunay et. al., 2000; Nguyên et al., 2001). O N-terminal de Yap1p contém um domínio bZip, que é conservado dentro da família AP-1. Um domínio rico em cisteína (CRD) no C-terminal de Yap1p desempenha papel importante no controle da localização intracelular de Yap1p. Normalmente este fator de transcrição ocorre tanto no núcleo quanto no citoplasma, porém sob condições de estresse oxidativo ele fica mais concentrado no núcleo. A localização do Yap1p é regulada primariamente através de um exportador nuclear Xpo1, homólogo de Crm1 de mamífero. A seqüência de exportação nuclear ("Nuclear export sequence – NES") do Yap1p está localizada no domínio C-terminal

altamente conservado, dentro do CRD. A remoção de CRD, mutação do NES, ou o tratamento com agentes oxidantes, diminui o reconhecimento do Yap1p pelo Xpo1, resultando no seu acúmulo nuclear, onde pode ativar a transcrição de genes alvo (Izawa et al., 1999; Delaunay et. al., 2000; Nguyên et al., 2001). Yap1p controla a indução de pelo menos 32 produtos gênicos, alguns dos quais modulados a nível transcricional (Lee et al., 1999). As mutantes deletadas Δ*yap1* são hipersensíveis ao estresse oxidativo, apresentam respostas adaptativas deficientes ao H_2O_2 e crescimento seriamente prejudicado na presença de H_2O_2, t-BOOH, diamida, dietilmaleato e cadmio (Schnell et al., 1992; Hirata et al., 1994; Kuge e Jones, 1994; Stephen et al., 1995).

5.3 Testes de sensibilidade de linhagens de *S. cerevisiae* ao tratamento com pro- e/ou antioxidantes.

Linhagens isogênicas deficientes em defesas antioxidantes têm sido utilizadas para o estudo do mecanismo de ação de agentes físicos e químicos que interferem com o estado redox celular (Brennan e Schiestl, 1998; Lee et al., 2001). Um método utilizado consiste em comparar a sensibilidade, ao tratamento com um agente físico (por ex. radiação) ou químico (por ex. pró/antioxidante), de diversas mutantes deficientes em enzimas antioxidantes, ou em um fator de transcrição sensível ao estado redox, como o Yap1p, ou ainda deficiente na síntese de GSH, a fim de avaliar a importância de cada defesa antioxidante celular na detoxificação do agente testado. Também é possível combinar um oxidante conhecido, como H_2O_2, t-BOOH e paraquat, com uma substância antioxidante ou com mecanismo desconhecido, e avaliar o efeito do tratamento da substância na modulação do estresse oxidativo. O aumento da viabilidade celular ao

tratamento estará sugerindo atividade protetora (antioxidante) e a diminuição da viabilidade a um efeito deletério (pró-oxidante) (Henriques et al., 2001).

Respostas adaptativas ao estresse oxidativo também podem ser encontradas em levedura. Células pré-tratadas com concentração sub-letal de um oxidante (H_2O_2, t-BOOH, paraquat) induz uma resposta protetora que permite que as mesmas sobrevivam a um tratamento com concentrações mais altas ou letais do oxidante (Kuge e Jones, 1994; Izawa et al., 1995; 1996; Grant et al., 1998). Esta resposta adaptativa não é restrita ao estresse oxidativo, sendo que em levedura a resposta adaptativa mais conhecida é a do choque térmico. Respostas similares também ocorrem para o estresse osmótico, agentes que provocam danos ao DNA e outros tipos de estresse. O pré-tratamento com um tipo de agente estressor pode também induzir resistência cruzada contra outro tipo de agente estressor (Park et al., 1998; Lee et al., 1999; Sugiyama et al., 2000). Esses estudos demonstram a complexidade de sistemas altamente regulados que evitam os danos celulares. A regulação desses sistemas em levedura pode ser mais complexo do que daqueles de organismos aeróbios, pois a levedura é um aeróbio facultativo (Maris et al., 2001).

6. ESTUDO DE MEMÓRIAS DE CURTA E LONGA DURAÇÃO

A memória é um processo dinâmico que pode ser dividido em três etapas principais: (1) aquisição da informação (2) consolidação e armazenamento, que é o processo de filtração e fixação progressiva da informação inicialmente adquirida, (3) evocação (“retrieval”) é o processo de resgate ou expressão dos eventos consolidados (Izquierdo, 1989; Izquierdo e Medina, 1997; Cahill et al., 2001). A conseqüência dos três processos envolvidos na memória seria a aprendizagem (aquisição de novas

memórias) e formação de um novo comportamento, ou modificação de um pré-existente. Pode-se definir memória como a conservação e evocação de informações adquiridas através de experiências vividas (Izquierdo e McGaugh, 2000). Os indivíduos apresentam capacidade de adaptação e modificação de seu comportamento quando expostos a novas experiências e a capacidade de aprender e recordar eventos depende de modificações induzidas no sistema nervoso pela percepção desses eventos (Izquierdo, 1989; Thompson e Kim, 1996; Markowitsch, 1997).

A capacidade de adquirir novas informações é uma das mais importantes funções do sistema nervoso e a expressão de memórias previamente adquiridas é crucial para a evolução das espécies (McGaugh, 2000). Evidências clínicas e experimentais há muito demonstraram que as memórias não consistem em um processo único, variando, por exemplo, em conteúdo e duração (McGaugh e Izquierdo, 2000). De acordo com seu conteúdo, as memórias são classificadas em declarativas ou explícitas (são conscientemente adquiridas e evocadas, podendo variar amplamente em sua duração) e procedurais ou implícitas (são adquiridas gradativamente e uma vez estabelecidas são evocadas de maneira inconsciente e constituem traços duradouros) (Thompson e Kim, 1996; Markowitsch, 1997). De acordo com o tempo durante o qual são armazenadas, as memórias também podem ser divididas em dois tipos principais: memórias de curta duração ("Short-term memory – STM") e memória de longa duração ("Long-term memory – LTM"). As STM são aquelas retidas dentro de alguns segundos até algumas horas após o aprendizado e as LTM são aquelas cuja consolidação é mais demorada e persistem dias, anos ou mesmo uma vida inteira (Izquierdo et al., 1999; Izquierdo et al., 1998a; Bianchin et al., 1999).

Acredita-se que o substrato neural das memórias, tanto de curta quanto de longa duração, resida em alterações nas conexões sinápticas entre neurônios de regiões cerebrais específicas e a natureza de tais modificações induzidas pela aquisição de novas memórias tem atraído muita atenção nas últimas décadas (Izquierdo e Medina, 1997; Roesler et al., 2000). Estudos dos mecanismos neurais das LTM demonstram que sua formação é um processo gradual que envolve substratos neuroanatômicos, celulares e moleculares específicos. LTM não são imediatamente adquiridas sob sua forma estável, sendo necessário serem consolidadas. Durante o período que se segue à sessão de aprendizado, enquanto as LTM são consolidadas, as STM são funcionais (Goelet et al., 1986; Izquierdo et al., 1998a; 1999). Estudos utilizando tratamentos farmacológicos específicos capazes de cancelar a formação de STM sem afetar a LTM para a tarefa de esquiva inibitória, demonstraram que memórias de curta e de longa duração são processadas em paralelo, compartilhando estruturas cerebrais e mecanismos celulares, porém de maneira independente (Izquierdo et al., 1998b; Izquierdo et al., 1999; Izquierdo e McGaugh, 2000).

Na tarefa de esquiva inibitória, o animal aprende a relacionar a descida de uma plataforma com um leve choque aplicado nas patas (Izquierdo, 1989; Roesler et al., 1998; Roesler et al., 2000). Com isso, numa segunda exposição à caixa de esquiva inibitória ele evita um comportamento inato de descer da plataforma para explorar a caixa. O aprendizado de EI envolve vários estímulos, incluindo percepção espacial e visual, sensibilidade à dor, acompanhados de um componente emocional amplamente modulado pela amígdala e por hormônios relacionados ao estresse (Izquierdo, 1989; Izquierdo e Medina, 1997; Walz et al., 2000). A EI é uma tarefa ideal para estudar processos de memórias iniciados durante e após o treino pois (1) em geral pode ser

aprendida com uma única sessão de treino; (2) não é um aprendizado inato; (3) a farmacologia envolvida na sua modulação é bastante conhecida.

O método principal de estudo consiste em administrar uma substância minutos antes da sessão treino ou logo após essa, na qual o animal é colocado sobre uma plataforma e o tempo de permanência (latência) é contado até que ele desça com todas as 4 patas a uma grade onde ele recebe um choque. Nas sessões de teste, o animal é recolocado na plataforma, porém não recebe choque, sendo que o tempo de latência em que o animal permanece na plataforma é cronometrado e usado como medida de retenção (Roesler et al., 1998; 1999a; 2000). A primeira sessão teste ocorre 1,5 h após o treino, a fim de medir a STM, e a segunda 24- 48 h após o treino, para medir a LTM. A retenção (memória) de curta e de longa duração da tarefa de EI pode ser prejudicada ou melhorada pela substância administrada; os efeitos observados nos dois tipos de memória são independentes, portanto, uma substância pode bloquear uma das memórias sem afetar a outra ou ainda apresentar efeitos de melhora numa e de prejuízo na outra (Izquierdo et al., 1999; Izquierdo e McGaugh, 2000).

As memórias de longa duração podem ser divididas em associativas e não associativas, dependendo do mecanismo requerido para a sua formação. As memórias associativas são baseadas na aquisição prévia de uma associação entre um evento específico e um estímulo. A tarefa de EI é um exemplo de aprendizado associativo (Izquierdo et al., 1997; Roesler et al., 1999b). Já as memórias não associativas são adquiridas quando a exposição repetida ou contínua a um novo estímulo modifica o comportamento inicial (Vianna et al., 2000). Uma das mais elementares tarefas de aprendizagem não associativa e não aversiva é a da habituação a um novo ambiente. Na exploração ao campo aberto, os animais são colocados em um campo aberto, um

compartimento que é semelhante a uma caixa de madeira (50 X 50 X 30 cm), com uma parede de vidro frontal, e o piso marrom dividido com linhas pretas em 12 quadrados iguais. O animal é observado durante 5 min nos quais ele pode explorar livremente a caixa. São contados o número de cruzamentos das linhas pretas ("crossings"), o número de vezes em que o animal levanta as duas patas dianteiras ("rearings"), o tempo de latência para o início da locomoção e o número de bolos fecais, durante os 5 min, e estas contagens são utilizadas como medida de locomoção, exploração, motivação e ansiedade (Roesler et al., 1998; 1999a; 2000). A habituação é medida pela diminuição da atitude exploratória que está associada ao número de "crossings" e "rearings" numa sessão teste realizada 24 h após a primeira exposição ao campo aberto (sessão treino) (Vianna et al., 2000).

7. ESTUDO DO COMPORTAMENTO ESTEREOTIPADO

O comportamento estereotipado é descrito como um comportamento no qual um indivíduo exibe atividade motora repetitiva, rítmica e invariável, e sem uma finalidade ou propósito real. Geralmente as informações sobre a base neurobiológica do comportamento estereotipado são obtidas pelo estudo em modelos animais do comportamento induzido por fármacos ou associado a um determinado ambiente (Powell et al., 1999; Presti et al., 2002). Este comportamento pode ser induzido em diversas espécies de mamíferos pela administração de agonistas dopaminérgicos, como APO, e fármacos que indiretamente afetam a transmissão dopaminérgica, como anfetamina. Uma injeção direta de dopamina ou de um agonista dopaminérgico no estriado induz comportamento estereotipado em ratos. Este comportamento pode ser inibido por antagonistas dopaminérgicos, como o haloperidol, ou pela inibição da

síntese de dopamina, usando α-metil-p-tirosina, ou ainda pela destruição de neurônios que contém dopamina com a neurotoxina 6-hidroxidopamina (6-OHDA) (Powell, et al., 1999; Presti et al., 2002). Em humanos, o comportamento estereotipado ocorre em desordens neurológicas e psiquiátricas como na esquizofrenia e na compulsão-obsessiva, na Doença de Parkinson, no autismo, entre outras, e está tipicamente associado com um aumento na atividade dopaminérgica (Goldman et al., 1998).

Este comportamento pode ser estudado em modelos que utilizam camundongos, no qual é definido por movimentos rápidos e repetitivos da cabeça e/ou membros dianteiros, que persistem por no mínimo 30 s, dentro do período de um minuto de observação. Geralmente, o animal é colocado numa caixa, após administração de uma substância, e observado de 10 em 10 min durante 1 min. O número de animais por caixa pode ser escolhido para otimizar o padrão específico do comportamento identificado como estereotipado (Battisti et al., 2000c).

8. OBJETIVOS

A apomorfina apresenta atividades farmacológicas de interesse para o tratamento da Doença de Parkinson e mais recentemente para o tratamento de disfunção erétil, além de ser utilizada na pesquisa das funções da via dopaminérgica e de novos fármacos para o tratamento de doenças neurodegenerativas e psiquiátricas. A capacidade de autoxidação confere à apomorfina propriedades de modular o estado redox celular, sendo que alguns de seus efeitos neurobiológicos e a genotoxicidade têm sido associados aos derivados da sua autoxidação.

8.1 Objetivo geral

Estudar as atividades genotóxica, antigenotóxica, citotóxica, antioxidante e comportamental de apomorfina (APO) e de um produto derivado da sua autoxidação 8-oxo-apomorfina-semiquinona (8-OASQ), isolado no Laboratório de Genotoxicidade da UFRGS, pelo Dr. Nikita Khromov-Borisov (Khromov-Borisov et al., 2000) (anexo I), utilizando diferentes sistemas biológicos.

8.2 Objetivos específicos

O presente trabalho teve os seguintes objetivos específicos

- Comparar o efeito mutagênico da APO com o produto de sua autoxidação 8-oxo-apomorfina-semiquinona (8-OASQ), utilizando as linhagens TA97, TA98, e TA102 de *Salmonella typhimurium* e as linhagens IC188 e IC203 de *Escherichia coli*, em presença e ausência de ativação metabólica.

- Determinar a atividade antigenotóxica de APO nas linhagens TA102, IC188/IC203, que detectam mutágenos oxidantes e ERO.

- Avaliar efeitos de proteção celular e citotóxicos de APO e de 8-OASQ em linhagens da levedura *Saccharomyces cerevisiae*, selvagem e isogênicas deficientes em genes envolvidos na resposta ao estresse oxidativo.

- Avaliar os danos ao DNA provocados por APO e 8-OASQ, utilizando o Ensaio Cometa, em amostras de cérebro total e sangue periférico de camundongos.

- Estudar o efeito de APO e de 8-OASQ sobre as diferentes fases da memória de esquiva inibitória, em ratos.

- Estudar o efeito de APO e de 8-OASQ sobre a atividade de ratos durante a exploração de um campo aberto.

- Verificar a indução de comportamento estereotipado de APO e de 8-OASQ em camundongos.

II – CAPÍTULO I

Apomorphine and its controversial effects: antioxidant, oxidant or mutagenic

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APOMORPHINE AND ITS CONTROVERSIAL EFFECTS: ANTIOXIDANT, OXIDANT OR MUTAGENIC.

Jaqueline N. Picada[a,b], Angel F. Maris[c], Karina Ckless[d], Mirian Salvador[e], Nikita N. Khromov-Borisov[f], João Antonio Pêgas Henriques[a]*

[a]Centro de Biotecnologia e Departamento de Biofísica, Universidade Federal do Rio Grande do Sul, UFRGS, Av. Bento Gonçalves 9500, Prédio 43421, Campus do Vale, Caixa Postal 15005, CEP 91501-970, Porto Alegre, RS, Brasil.

[b]Departamento de Farmácia, ULBRA, Canoas, RS, Brasil

[c]Departamento de Bioquímica, UFSC, Florianópolis, SC, Brasil

[d]Departamento de Farmacologia e Departamento de Ciências Farmacêuticas, UFSC; Florianópolis, SC, Brasil.

[e]Instituto de Biotecnologia, UCS, Caxias do Sul, RS, Brasil.

[f]Biological Institute of St.Petersburg University, Saint-Petersburg, Russia.

*Corresponding author. Fax: +21 51 3316 6084

e-mail address: Pegas@dna.cbiot.ufrgs.br (JAP Henriques)

ABSTRACT

Apomorphine (APO) is a potent dopamine agonist which has been used in the theraphy of Parkinson's disease (PD). In May of 2001 apomorphine HCl, under the trade name Uprima®, was approved for the treatment of erectile dysfunction, increasing significantly the number of potential users of this drug. Nevertheless, there is still a certain controversy about the safety of such a large increase in APO prescription, especially since the relationship between benefits and adverse effects, which is good in the case of Parkinson's disease, is doubtful for the treatment of erectile dysfunction. Previous studies demonstrated that this compound acts either as antioxidant or as pro-oxidant. We investigated mutagenicity, antimutagenicity, cytotoxicity, pro- and antioxidant effects of apomorphine (APO) and its oxidation derivative, 8-oxo-apomorphine-semiquinone (8-OASQ), by means of the *Salmonella*/microsome assay, WP2 Mutoxitest and sensitivity assay in *Saccharomyces cerevisiae* WT strains and in Δ*yap1,* Δ*gsh1,* Δ*ctt1,* Δ*sod1,* Δ*sod2* strains. In the absence of S9 mix both compounds induced frameshift mutation in TA98 and TA97 *S. typhimurium* strains, with 8-OASQ being up to two times more mutagenic. However for strains which detect oxidative mutagens, 8-OASQ acted as a mutagen while APO was an antimutagen, inhibiting H_2O_2 and t-BOOH-induced mutagenicity in TA102 *S. typhimurium* and WP2-derived *E. coli* strains. The S9 mix inhibited all mutagenic effects, probably either by retarding oxidation of APO or by conjugation of APO and its autoxidation products, such as 8-OASQ, to proteins. In sensitivity assays with *S. cerevisiae*, APO was only clearly cytotoxic to some strains at higher doses, whereas 8-OASQ dose-dependently sensitized all the strains, mainly the catalase (*ctt1*), superoxide dismutase (*sod1*) and *yap1* deletion mutants, suggesting that 8-OASQ cytotoxicity towards *S. cerevisiae* results from its pro-oxidant properties. APO also tended to protect *S. cerevisiae* strains against oxidative

damage induced by high concentrations of H_2O_2 and t-BOOH, while 8-OASQ enhanced pro-oxidant effects and induced adaptation responses to these agents.

Keywords: Apomorphine, oxidative stress, mutagenicity, antimutagenicity, yeast, Ames *Salmonella*/microsome assay.

1. INTRODUCTION

Apomorphine [APO; 4*H*-Dibenzo(*de,g*)quinoline-10,11-diol, 5,6,6a,7-tetrahydro-6-methyl-(*R*)] is considered to be a classical mixed type dopamine D_1 and D_2 receptor agonist. It has been used in the therapy of Parkinson's disease (PD) and more recently for the treatment of erectile dysfunction [1-5]. Neuroprotection induced by APO has been demonstrated using experimental models of chronic degenerative diseases [6]. In particular, recent biochemical and molecular evidence indicates that the onset and progression of neuronal damage observed both in PD patients and in different models of experimental Parkinsonism are mainly due to the generation of free radicals and oxidative species [7]. Neuroprotective effects induced by APO appear to be based on its chelating and free radical scavenging properties and do not seem to be related to its dopamine agonist activity [8-13].

Like other catechols (e.g. dopamine), APO is unstable in aqueous solution and is rapidly oxidized to various derivatives of quinolindione, resulting in solutions of yellowish brown to emerald green coloration [14,15]. Only few investigations of deleterious and/or protective effects of APO both *in vivo* and *in vitro* and their association with its redox transformations have been performed and the mechanism of toxicity of the alkaloid remains unclear. The oxidation-related properties of APO lead to activities that appear paradoxical: APO may act as an antioxidant, or as a pro-oxidant (reviewed in [2]). Ubeda et al. [16] reported that APO can act as a pro-oxidant, stimulating damages to DNA and deoxyribose

degradation induced by Fe^{3+} and Cu^{2+} by a mechanism related to the generation of superoxide anion radicals. On the other hand, APO has been shown to inhibit membrane lipid peroxidation *in vitro* and *in vivo* [17].

Low concentrations of APO have been found to protect rat pheochromocytoma (PC12) cells from the toxic effects of hydrogen peroxide and 6-hydroxydopamine, while high concentrations were cytotoxic and caused cell death in the presence of hydrogen peroxide [18]. More recently, El-Bachá et al. [19,20] showed APO to promote necrosis in rat glioma C6 cells through the formation of reactive oxygen species (ROS), quinones and a melanin-like pigment during autoxidation. Both apomorphine autoxidation and cell damage were prevented by thiols. All proteins appeared green after incubation with APO, suggesting the formation of a non-specific apomorphine-protein conjugate.

Little is known about the genotoxic effect of this alkaloid. It has been shown to induce frameshift mutations in *Salmonella typhimurium* TA1537 and the mutagenic action was attributed to autoxidation derivatives [21]. Clastogenic activity of APO has been demonstrated a in Chinese hamster lung cell line (CHL), where APO induced 10% aberrant cells at 12.5 μg/ml in the absence of S9 metabolic activation mixture (S9 mix) [22]. Treatment with S9 mix significantly reduced clastogenicity of APO.

All these data suggest a relationship between the oxidation state of APO and its pro- and/or antioxidant properties, in addition to its genotoxic effects [1,19-22]. Recently, we described the isolation of an apomorphine autoxidation semiquinone, 8-oxo-apomorphine-semiquinone (8-OASQ) [23]. The influence this autoxidation product of APO on its frameshift mutagenic activity was assessed by testing unoxidized APO and 8-OASQ in TA97 and TA98 *S. typhimurium* frameshift mutants in the *Salmonella*/microsome assay [24,25]. In order to evaluate the oxidative mutagenic and/or antimutagenic effects of APO

and 8-OASQ we used three bacterial strains recommended for the screening of oxidizing mutagens, *Escherichia coli* IC203 (WP2 *uvrA oxyR*/pKM101), deficient in the oxidative stress response transcription factor OxyR, and its *oxyR*$^{+}$ parent IC188 (WP2 *uvrA*/pKM101) [26-28], and *Salmonella typhimurium* TA102 [24,25,29]. The cytotoxic, pro-oxidant, antioxidant and cross-adaptive effects of APO and 8-OASQ were assessed by means of a survival assay in *Saccharomyces cerevisiae* WT strains and in Δ*yap1,* Δ*gsh1,* Δ*ctt1,* Δ*sod1,* Δ*sod2* deletion mutants.

2. MATERIALS AND METHODS

Chemicals

Apomorphine hydrochloride (APO) (*CAS* Registry Number [314-19-2]) was purchased from Merck KGaA (Darmstadt, Germany) and 8-oxo-apomorphine-semiquinone (8-OASQ) was isolated as described in [23].

Strains

Salmonella typhimurium TA97, TA98 and TA102, described in [24], and *Escherichia coli* IC203 (WP2 *uvrA oxyR*/pKM101) and its *oxyR*+ parent IC188 (WP2 *uvrA*/pKM101), described in [26], were kindly provided by B. M. Ames (University of California, Berkeley, CA, USA) and M. Blanco (Instituto de Investigaciones Citológicas, Valencia, Spain), respectively. Strains of *Saccharomyces cerevisiae* employed in this study are given in Table 1.

Table 1. Description of the *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source
EG103 (WT)	*MATα leu2-3 112 his3Δ1 trp1-289 ura3-52* GAL^+	E. B. Gralla, Los Angeles
EG118 (Δ*sod1*)	*MATα leu2-3 112 his3Δ1 trp1-289 ura3-52* GAL^+ *sod1ΔA::URA3*	E. B. Gralla, Los Angeles
EG223 (Δ*ctt1*)	*MATα leu2-3 112 his3Δ1 trp1-289 ura3-52* GAL^+ *ctt1Δ::TRP1*	E. B. Gralla, Los Angeles
EG110 (Δ*sod2*)	*MATα leu2-3 112 his3Δ1 trp1-289 ura3-52* GAL^+ *sod2Δ::TRP1*	E. B. Gralla, Los Angeles
EG213 (Δ*ctt1*Δ*sod1*)	*MATα leu2-3 112 his3Δ1 trp1-289 ura3-52* GAL^+ *ctt1Δ::TRP1 sod1ΔA::URA3*	E. B. Gralla, Los Angeles
EG133 (Δ*sod1*Δ*sod2*)	*MATα leu2-3 112 his3Δ1 trp1-289 ura3-52* GAL^+ *sod1ΔA::URA3 sod2Δ::TRP1*	E. B. Gralla, Los Angeles
YPH98/ (WT)	*MATa ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52*	P. Hieter, Baltimore
Δ*snq3*/Δ*yap1*	*MATa ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 Snq3::URA3*	M. Brendel, Frankfurt
Q1 (*GSH1*)	*MATa ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52* rho^o	M. Grey, Frankfurt
Q2 (Δ*gsh1*)	*MATa ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52* rho^o *gsh1::URA3*	*M. Grey, Frankfurt*

S9 fraction

The S9 fraction, prepared from livers of Sprague-Dawley rats pre-treated with the polychlorinated biphenyl mixture Aroclor 1254, was purchased from Moltox (Annapolis, MD, USA). The S9 metabolic activation mixture (S9 mix) was prepared according to [24].

Salmonella/microsome mutagenicity assay

Mutagenicity was assayed by the preincubation procedure proposed by [24,25]. APO and 8-OASQ were dissolved in DMSO immediately prior to use. 100 µl of test bacterial cultures (1-2 x 10^9 cells/ml) were incubated in the dark at 37 ^oC with different amounts of APO or 8-OASQ (10, 20, 40, 60 and 80 µg/plate) in the presence or absence of S9 mix for 20 min, without shaking. Subsequently, 2 ml of soft agar (0.6% agar, 0.5% NaCl, 50 µM histidine, 50 µM biotin, pH 7.4, 45 ^oC) were added to the test tube and poured immediately onto a plate of minimal agar (1.5% agar, Vogel-Bonner E medium, containing 2% glucose). Aflatoxin B_1 (AFB1, 0.5 µg/plate) was used as positive control for all strains in the metabolic assay with S9 mix. In the absence of S9 mix, the positive control was 4-nitroquinoline-oxide (4NQO, 0.5 µg/plate). The plates were incubated in the dark at 37 ^oC for 48 h before counting the revertant colonies. All assays were done at least in duplicate.

Escherichia coli mutagenicity assay – WP2 Mutoxitest

Mutagenicity was performed according to [26] with a modified pre-incubation procedure [30]. 100 µl of a fresh overnight culture were incubated without shaking at 37 ^oC with different amounts of APO or 8-OASQ (10, 20, 40, 60 µg/plate), in the presence or absence of S9 mix (prepared according to [24]) for 20 min, in tubes protected from light. 2 ml of molten top agar at 45 ^oC (supplemented with 0.05 mM tryptophan) were added to these tubes and poured on to minimal ET4 plates (solid minimal E4 medium contained 15 g Difco

agar and 4 g glucose per litre of Vogel-Bonner E medium). These were incubated for 48 h at 37 ^{o}C. Tert-butylhydroperoxide (t-BOOH, 50 μg/plate) was used as positive control.

Antimutagenicity assay

S. typhimurium TA102 and *E. coli* IC203 and IC188 were used to assess APO antimutagenicity against the oxidative mutagens t-BOOH and H_2O_2. The pre-incubation procedure was as follows: APO was incubated with the culture at 37 ^{o}C without shaking, in the presence or absence of S9 mix, for 20 min. An oxidative mutagen was then added and the mixture further incubated at 37 ^{o}C for 20 min, followed by plating on the appropriate media. Plates were incubated in the dark at 37 ^{o}C for 48 h before counting the revertant colonies. All assays were done in triplicate.

Sensitivity assay of S. cerevisiae strains.

YPD-grown yeast cells from the early stationary phase were re-inoculated at an appropriate cell density in fresh YPD media containing 5% glucose (glucose repressing conditions) and grown for 4 h at 30 ^{o}C to a density of 1-2 x 10^7 cells/ml. To evaluate sensitivity to APO and 8-OASQ, cultures were exposed to concentrations varying from 40 μg/ml to 400 μg/ml and incubated under growth conditions for 1 h. For cross-adaptation experiments, cells were pre-treated with non-cytotoxic concentrations of APO (20 and 60 μg/ml) or 8-OASQ (5 and 20 μg/ml) and incubated for 1 h. Pre-treated cells were then challenged with a lethal concentration of H_2O_2 or t-BOOH and incubated for another hour. To assess the antioxidant or pro-oxidant effects, the cells were co-treated with APO or 8-OASQ plus each oxidant (H_2O_2, t-BOOH) at the same concentrations used for the cross-adaptation experiments.

Cells were appropriately diluted and plated in triplicate on solid YPD (1% yeast extract, 2% peptone, 2% glucose, and 2% agar). After 3 days, colony-forming units were counted. All tests were done in triplicate.

Data analysis

Mutagenicity data were analyzed with the *Salmonel* software [31]. A compound was considered positive for mutagenicity only when: (a) the number of revertants was at least double the spontaneous yield (MI ≥ 2; MI= mutagenic index: n^{o} of induced colonies in the sample/ n^{o} of spontaneous in the negative control); (b) a significant response for analysis of variance ($p \leq 0.05$) was found; and (c) a reproducible positive dose-response ($p \leq 0.01$) was present, evaluated by the *Salmonel* software [32].

The percentage of inhibition of each antimutagen against each mutagen was calculated as: % Inhibition = [1- (B/A)] X 100, where A represents the number of revertants on the plate containing mutagen only and B represents the number of revertants on the plate containing mutagen and antimutagen. The number of spontaneous revertants on the negative control plate was subtracted from each of A and B [33].

The dose inhibiting 50% of mutagenicity can be calculated from the dose-response curve and can be used as an indicator of antimutagenic potency.

Data from the cross-adaptation assay and detection of anti or pro-oxidant effects in *S. cerevisiae* were subjected to Dunnett's multiple comparison test; $p \leq 0.05$ was considered to indicate statistical significance.

3. RESULTS

Induction of frameshift mutations in bacteria:

The mutagenicity of APO and 8-OASQ in the frameshift mutation detecting TA98 and TA97 strains, in the presence or absence of metabolic activation, is shown in Table 2. In the absence of S9 mix, clear mutagenic effects of both compounds were observed in *Salmonella typhimurium* TA98 and TA97. At an APO dosage of 60 μg/plate the Mutagenicity Index (MI) in TA98 and TA97 reached values of 2.1 and 3.0, respectively, while 8-OASQ induced more than twice this level, showing MI of 5.4 and 8.2 at 60 μg/plate for TA98 and TA97, respectively (Table 2). When performed under metabolic activation, APO and 8-OASQ lost their mutagenic activities at the dose range tested (Table 2).

Table 2. Induction of *his*$^+$ revertants in *S. typhimurium* frameshift strains by apomorphine (APO) and 8-oxo-apomorphine semiquinone (8-OASQ), with and without metabolic activation (S9 mix).

		TA98				TA97			
		-S9		+S9		-S9		+S9	
Substance	Dose µg/plate	Rev/plate[a]	MI[b]	Rev/plate	MI	Rev/plate	MI	Rev/plate	MI
PC[c]	0.5	381±8	10.6	770±123	17.5	613±47	6.3	510±13	3.6
NC[d]		36±4		44±9		98±22		140±6	
APO	20	48±5	1.3	48±10	1.1	174±17*	1.8	177±4	1.3
	40	55±9	1.5	51±13	1.1	249±18**	**2.5**	208±16	1.5
	60	76±9**	**2.1**	60±11	1.4	291±22**	**3.0**	205±27	1.5
	80	86±2**	**2.4**	64±14	1.4	397±21**	**4.1**	241±38*	1.7
PC	0.5	139±25	7.7	948±103	16.3	459±41	6.3	309±45	3.6
NC		18±4		58±7		73±8		85±11	
8-OASQ	10	39±10	2.1	79±3*	1.4	227±22**	**3.1**	89±4	1.0
	20	51±3**	**2.8**	72±14	1.2	347±23**	**4.8**	115±9*	1.4
	40	64±6**	**3.5**	80±7*	1.4	456±34**	**6.2**	143±10**	1.7
	60	98±6**	**5.4**	75±6	1.3	597±34**	**8.2**	150±15**	1.8

[a]Number of revertantes/plate: mean values at least of two experiments ± SD.
[b]MI mutagenic index: n^o of *his*$^+$ induced in the sample / n^o of spontaneous *his*$^+$ in the negative control.
[c]PC positive control: (-S9) 4-nitroquinoline 1-oxide; (+S9) aflatoxin B1.
[d]NC negative control: dimethyl sulfoxide (DMSO, 10µl) used as a solvent for APO and 8-OASQ.
* $p<0.05$; ** $p<0.01$ (ANOVA).

Induction of oxidative mutagenesis in bacteria:

With the use of the oxidant sensitive *E. coli* IC188 (WP2 *uvrA*/pKM101) and its derivative that is sensitive to more reactive oxygen species (ROS), strain IC203 (WP2 *uvrA oxyR*/pKM101), we found a very pronounced mutation induction by 8-OASQ starting at 40 μg and 20 μg/plate, respectively, in the absence of the S9 mix (Table 3). The lack of effect for the *S. typhimurium* strain TA102 (Table 3), also considered to be ROS sensitive [25,29], was noteworthy. For APO the results are almost the reverse. In the absence of metabolic activation there is not an increase, but actually a decrease over spontaneous mutation rate of strains IC188 and IC203 (Table 3). Paradoxically, TA102 indicated a slight tendency for a dose-dependent increase in mutation; however, this does not classify APO as mutagenic in this test, since the mutagenic index (MI) never reaches more than 1.5 (Table 3). In the presence of S9 mix (Table 4), the mutagenic effects of 8-OASQ were completely inhibited and APO, again, did not induce mutagenesis.

Table 3. Mutagenic effects of apomorphine (APO) and 8-oxo-apomorphine-semiquinone (8-OASQ) in bacterial strains detecting oxidative mutagens, without metabolic activation (S9 mix).

		IC188		IC203		TA102	
Substance	Dose μg/plate	Rev./plate[a]	MI[b]	Rev./plate	MI	Rev./plate	MI
PC[c]	50	654±25	4.0	1559±53	14.0	1583±29	4.0
NC[d]		135±11		132±4		333±25	
APO	10	135±22	1.0	135±4	1.0	371±8	1.1
	20	110±6	0.8	107±7	0.8	407±33	1.2
	40	94±17	0.7	85±23	0.6	416±60	1.2
	60	74±0.6	0.5	97±4	0.7	498±11**	1.5
NC		165±5		110±3		392±27	
8-OASQ	10	207±27	1.3	171±28*	1.6	419±23	1.1
	20	311±25**	1.9	240±39*	**2.2**	407±26	1.0
	40	429±21**	**2.6**	447±81**	**4.1**	393±16	1.0
	60	528±52**	**3.2**	650±83**	**5.9**	375±13	1.0

[a]Number of revertantes/plate: mean values at least of two experiments ± SD
[b]MI= mutagenic index: n°. of induced colonies in the sample/ n° of spontaneous in the negative control.
[c]PC: positive control: tert-butylhydroperoxide (t-BOOH).
[d]NC: negative control: dimethyl sulfoxide (DMSO, 10 μl) used as a solvent for APO and 8-OASQ.
* $p<0.05$; ** $p<0.01$ (ANOVA).

Table 4. Mutagenic effects of apomorphine (APO) and 8-oxo-apomorphine-semiquinone (8-OASQ) in bacterial strains detecting oxidative mutagens, with metabolic activation (S9 mix).

		IC188		IC203		TA102	
Substance	Dose μg/plate	Rev./plate[a]	MI[b]	Rev./plate	MI	*Rev./plate*	MI
PC[c]	50	483±41	3.5	1417±74	13.6	1574±126	4.6
NC[d]		110±9		144±12		316±18	
APO	10	118±8	1.1	141±4	1.0	353±62	1.1
	20	110±13	1.0	142±6	1.0	388±7	1.2
	40	113±9	1.0	156±14	1.1	368±21	1.2
	60	119±27	1.1	160±17	1.1	368±17	1.2
NC		138±8		104±5		340±13	
8-OASQ	10	141±16	1.0	113±7	1.1	392±24	1.2
	20	127±5	0.9	101±22	1.0	432±37*	1.3
	40	107±10	0.8	103±16	1.0	435±36*	1.3
	60	119±23	0.9	117±26	1.1	449±20**	1.3

[a]Number of revertantes/plate: mean values at least of two experiments ± SD
[b]MI= mutagenic index: n°. of induced colonies in the sample/ n° of spontaneous in the negative control.
[c]PC: positive control: tert-butylhydroperoxide (t-BOOH).
[d]NC: negative control: dimethyl sulfoxide (DMSO, 10 μl) used as a solvent for APO and 8-OASQ.
* $p<0.05$; ** $p<0.01$ (ANOVA).

Protection against oxidants in bacteria:

The ability of APO to protect against oxidative mutagens was analyzed using the three oxidant sensitive bacteria strains, *E. coli* IC188, IC203, and *S. typhimurium* TA102. As model oxidants, we used hydrogen peroxide (H_2O_2, 25 and 100 μg/plate) and the organic hydroperoxide terc-butylhydroperoxide (t-BOOH, 25 and 100 μg/plate). APO was indeed antimutagenic, inhibiting by up to 90% the mutagenicity induced by t-BOOH and almost abolishing the mutagenic effect of H_2O_2, in the absence of S9 mix (Table 5). Dose-dependent values were obtained for all strains; however the values differed by about 20% between strains.

In the presence of S9 mix, H_2O_2 did not induce mutagenesis in strains IC203 and IC188 (Table 6). This was probably due to catalase activity present in the liver homogenate [27], which did not permit the evaluation of antimutagenic activity of APO. In TA102, in spite of the presence of S9 mix, H_2O_2 induced mutagenesis, which was inhibited by APO up to 50%. The mutagenicity induced by t-BOOH was not much affected by the S9 mix, which however reduced the antimutagenic activity of APO from around 80% (without S9 mix) to about 40% (Table 6).

Table 5. Effects of apomorphine (APO) on induced mutagenicity by t-butylhydroperoxide (t-BOOH) and hydrogen peroxide (H_2O_2), without metabolic activation (S9 mix).

	IC188			IC203		TA102		
Substances	Doses µg/plate	Rev. /plate[a]	% I[b]	Rev./plate	% I	Doses µg/plate	Rev./plate	% I
t-BOOH + APO	25+0	782±7		1314±25		100+0	2707±139	
	25+10	738±20	6.5	1239±13	6.4	100+10	1771±130**	39.4
	25+20	751±117	4.6	1019±76**	25.0	100+20	1151±113**	65.5
	25+40	597±56**	27.4	946±142**	31.2	100+40	815±21**	79.6
	25+60	354±27**	63.5	572±23**	63.0	100+60	478±53**	93.8
	25+80	237±33**	80.9	472±12**	71.5			
[c]NC		108±11		136±9			330±20	
H_2O_2 + APO	100+0	311±38		603±36		25+0	1107±72	
	100+10	254±25*	27.8	431±14**	37.0	25+10	1000±39	12.9
	100+20	191±10**	58.5	391±61**	45.6	25+20	555±84**	66.7
	100+40	150±7**	78.5	314±26**	62.2	25+40	334±14**	93.5
	100+60	141±3**	82.9	80±9**	112.5	25+60	298±38**	97.8
NC		106±16		138±12			280±30	

[a]Number of revertantes/plate: mean values at least of two experiments ± SD.
[b]I= % Inhibition= [1-(B/A)] x 100, where A represents the number of revertants on the plate containing mutagen only and B represents the number of revertants on the plate containing mutagen and antimutagen. The number of revertants on the NC plate was subtracted from each of A and B.
[c]NC negative control: dimethyl sulfoxide (DMSO, 10µl) used as a solvent for APO and 8-OASQ.
* $p<0.05$; ** $p<0.01$ (ANOVA).

Table 6. Effects of apomorphine (APO) on induced mutagenicity by t-butylhydroperoxide (t-BOOH) and hydrogen peroxide (H_2O_2), with metabolic activation (S9 mix).

		IC188		IC203			TA102	
Substances	Doses µg/plate	Rev./plate[a]	% I[b]	Rev./plate	% I	Doses µg/plate	Rev./plate	% I
t-BOOH + APO	25+0	825±16		1418±74		100+0	2787±330	
	25+10	790±24	5.1	1239±13**	14.2	100+10	2631±80	6.3
	25+20	767±55	8.4	1173±64**	19.5	100+20	2199±7**	23.6
	25+40	723±30	14.8	1174±55**	19.4	100+40	2020±91**	30.8
	25+60	635±75**	27.5	1091±48**	26.0	100+60	1932±72**	34.3
	25+80	506±27**	46.2	887±35**	42.2			
[c]NC		135±13		161±14			294±5	
H_2O_2 + APO	100+0	128±8		190±4		25+0	579±2	
	100+10	119±12		176±19		25+10	545±28	11.6
	100+20	113±15		186±15		25+20	537±25	14.3
	100+40	108±18		187±20		25+40	476±27**	35.2
	100+60	108±17		224±21		25+60	413±48**	56.7
NC		92±5		134±12			286±16	

[a]Number of revertantes/plate: mean values at least of two experiments ± SD.
[b]I= % Inhibition= [1-(B/A)] x 100, where A represents the number of revertants on the plate containing mutagen only and B represents the number of revertants on the plate containing mutagen and antimutagen. The number of revertants on the NC plate was subtracted from each of A and B.
[c]NC negative control: dimethyl sulfoxide (DMSO, 10µl) used as a solvent for APO and 8-OASQ.
* $p<0.05$; ** $p<0.01$ (ANOVA).

Sensitivity to APO and 8-OASQ in Saccharomyces cerevisiae WT and antioxidant-deficient strains:

Cells of *S. cerevisiae* wild-type (WT) and isogenic mutant strains lacking antioxidant defenses (Table 1) were treated with APO and 8-OASQ for 1 h during exponential phase growth in glucose-repressing conditions, where the cells show high sensitivity to oxidative stress [34,35]. For strains with the EG103 background (Table 1), there was an obvious difference in sensitivity between APO and 8-OASQ for all, including the WT strain (Fig.1A and C). APO only induced significant effects at the higher doses (Fig.1A), whereas 8-OASQ severely affected survival at 4 to 8 times lower doses (Fig.1C). The Δ*ctt1* and Δ*sod1* deletion strains showed similar sensitivities and were the most sensitive single mutant strains to 8-OASQ (Fig.1C); both double mutants, Δ*ctt1*Δ*sod1* and Δ*sod1*Δ*sod2*, had higher sensitivity than the respective single mutants, indicating a non-epistatic effect of catalase and superoxide dismutase deficiency for detoxification of 8-OASQ. The least sensitive strain was the Δ*sod2* single mutant, which behaved like the WT strain (Fig.1C).

Among the strains with the YPH98 background (Table 1), the Δ*gsh1* strain was more sensitive to both compounds than both WT strains (Fig.1B and D). However, despite the higher sensitivity of all strains to 8-OASQ than to APO, the most dramatic loss in viability to the former was for the Δ*yap1* mutant (Fig.1D).

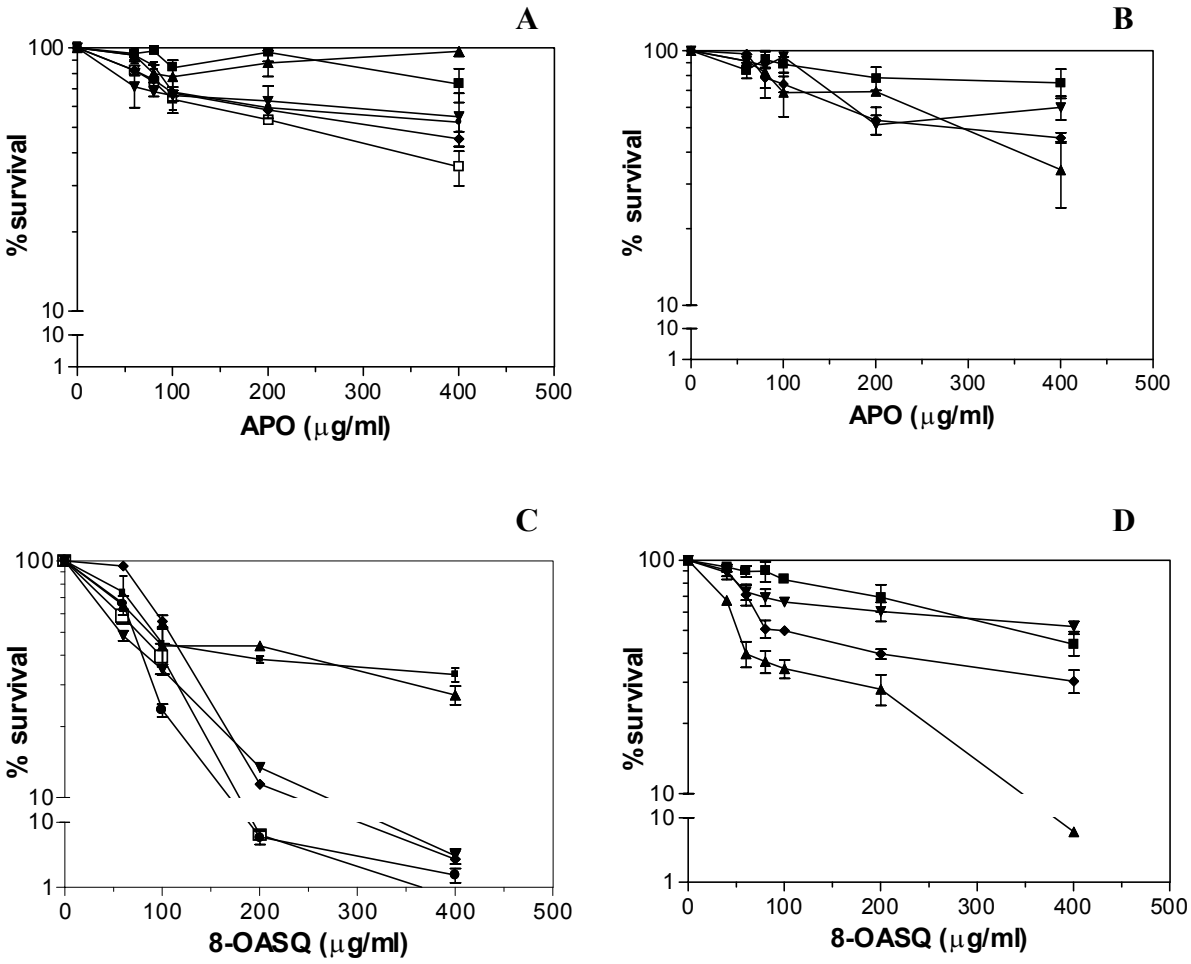

Figure 1. Sensitivity of cells in the exponential phase of growth to APO (**A-B)** and 8-OASQ **(C-D).** Left: EG103 (WT) (■) and its isogenic derivative strains: Δ*sod1* (▼); Δ*sod2* (▲); Δ*ctt1* (♦); Δ*sod1*Δ*sod2* (●); Δ*ctt1*Δ*sod1* ( ). Right: YHP98 (WT) (■); Δ*yap1* (▲); Q1 (WT *rho*^{0}) (▼); Q2 (Δ*gsh1 rho*^{0}) (♦). Cells were grown in YPD medium to exponential phase and 1 ml aliquots were challenged with various concentrations of APO or 8-OASQ for 1h at 30^{o}C. Cells were diluted in water and plated to assess survival.

***Protective or deleterious effects of APO and 8-OASQ against hydroperoxide induced damage*:**

APO showed protective effects at the non-cytotoxic concentrations of 20 and 60 μg/ml for most of the EG103 isogenic strains in both co- and pre-treatment assays, using H_2O_2 and t-BOOH as oxidants (Fig.2). Similar results were obtained for YPH98/Q1 isogenic strains, with the exception of APO prior to H_2O_2 challenge, which had no effect (Fig.3). In contrast, 8-OASQ was highly deleterious in the co-treatment with H_2O_2 and t-BOOH for some of the EG103 isogenic strains (Fig.4A and C) and the YPH98 derived Δ*yap1* and Δ*gsh1* mutants (Fig.5C). Co-treatment with H_2O_2 plus 8-OASQ also significantly decreased the percentage survival for all YPH98/Q1 isogenic strains (Fig.5A). Curiously, a significant increase in viability after H_2O_2 challenge was seen in the pre-treatment with the non-cytotoxic concentrations of 8-OASQ (Figs.4B and 5B) for almost all the strains except for the Δ*sod2* (Fig.4B) and the Δ*yap1* (Fig.5B) mutants. For t-BOOH, the pre-treatment procedure with 8-OASQ also induced some increase in survival for the EG103 isogenic strains (Fig.4D) but none for the YPH98/Q1 strains (Fig.5D).

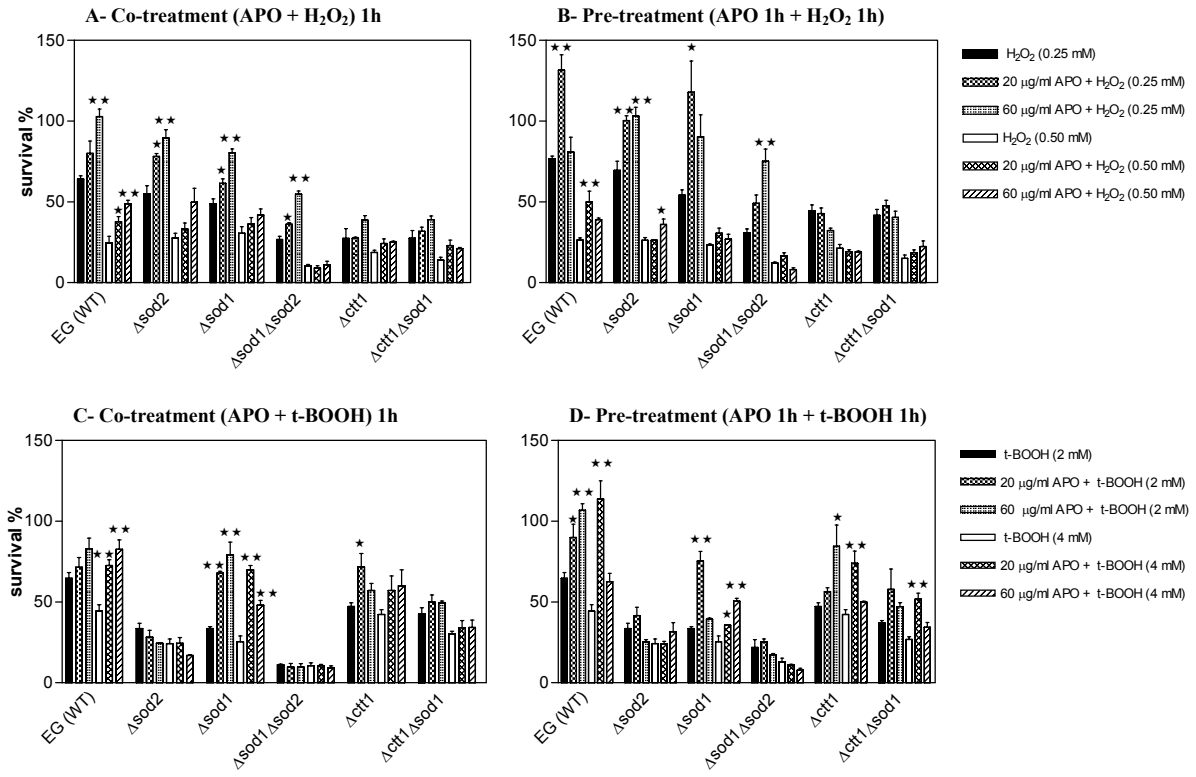

Figure 2. Effect of non-cytotoxic concentrations of APO on survival to oxidants H_2O_2 and t-BOOH (lethal concentrations) in EG103 isogenic yeast strains. A) co-treated with APO and H_2O_2 for 1h; B) pre-treated with APO for 1h then challenged with H_2O_2; C) co-treated with APO and t-BOOH for 1h; D) pre-treated with APO for 1h then challenged with t-BOOH. Percentage survival is expressed relative to untreated control cultures (100%). Values shown are the mean of at least three determinations. * $p<0.05$; ** $p<0.01$ indicate significant increase in survival % compared to the oxidant-treated samples (ANOVA, Dunnet's test).

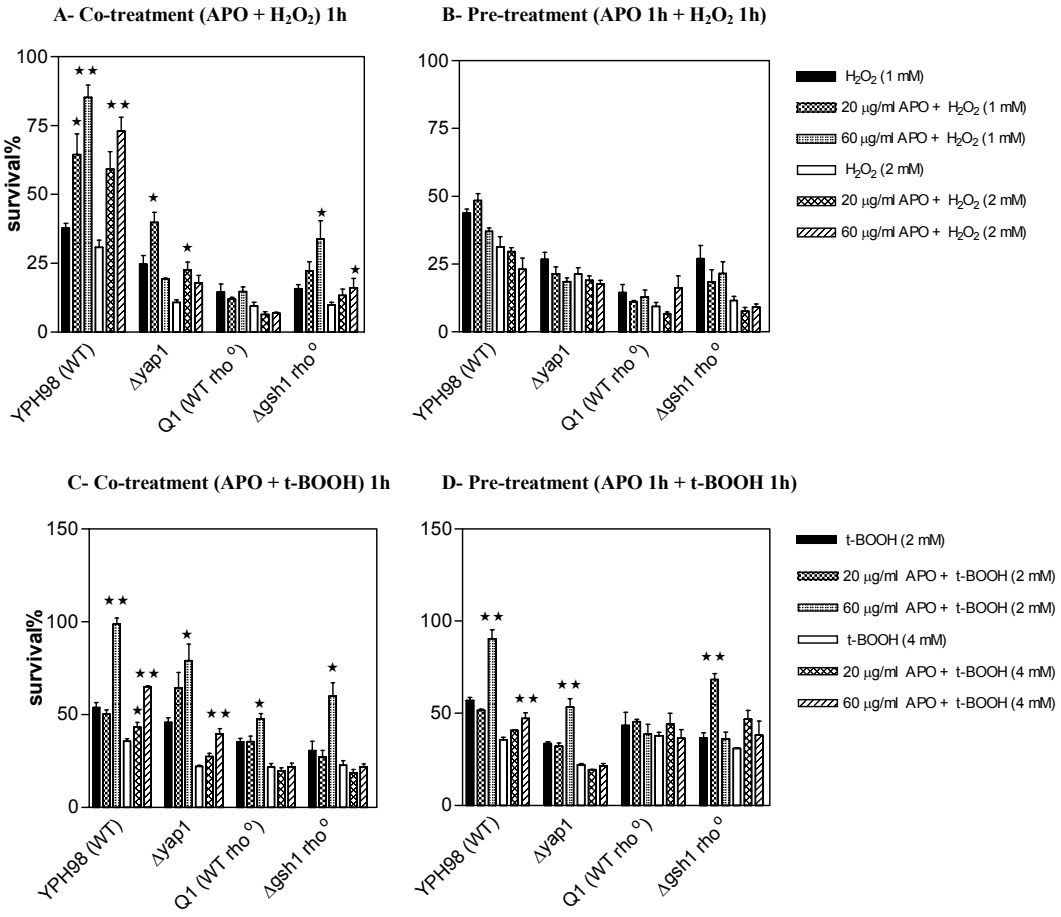

Figure 3. Effect of non-cytotoxic concentrations of APO on survival to oxidants H_2O_2 and t-BOOH (lethal concentrations) in YPH98/Q1 isogenic yeast strains. A) co-treated with APO and H_2O_2 for 1h; B) pre-treated with APO for 1h then challenged with H_2O_2; C) co-treated with APO and t-BOOH for 1h; D) pre-treated with APO for 1h then challenged with t-BOOH. Percentage survival is expressed relative to untreated control cultures (100%). Values shown are the mean of at least three determinations. * $p<0.05$; ** $p<0.01$ indicate significant increase in survival % compared to the oxidant-treated samples (ANOVA, Dunnet's test).

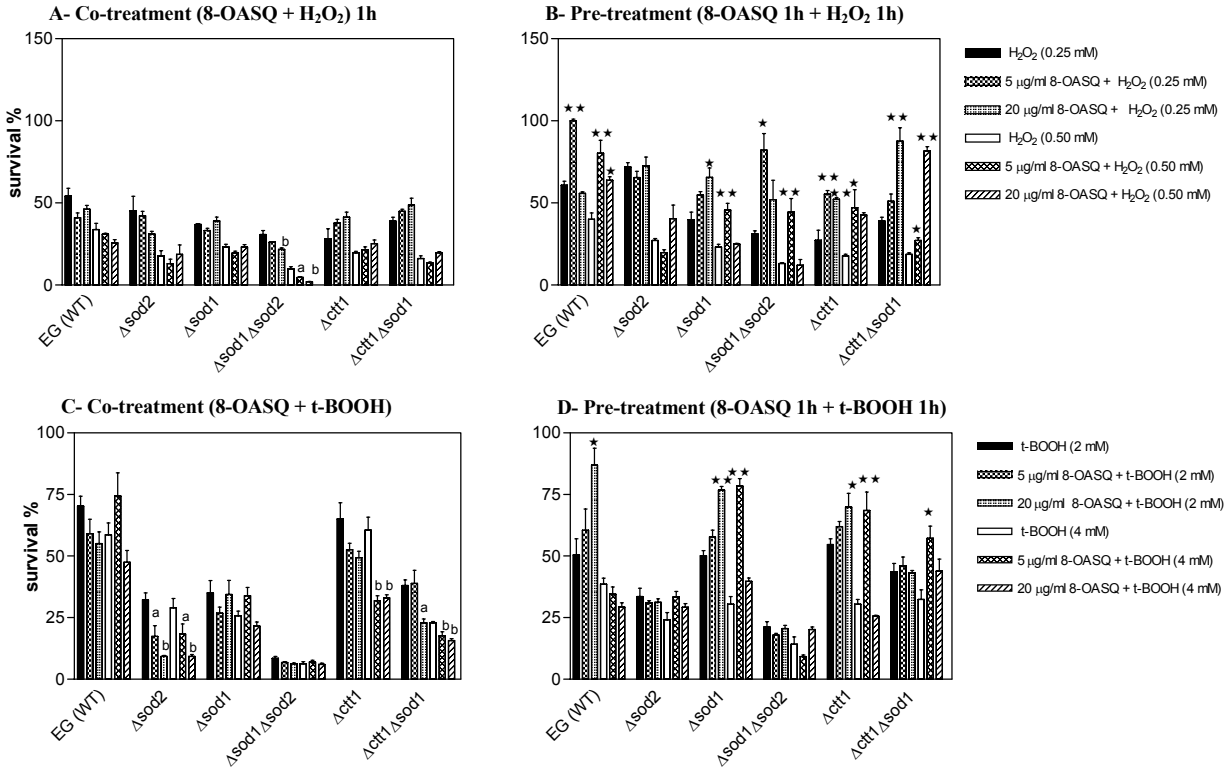

Figure 4. Effect of non-cytotoxic concentrations of 8-OASQ on survival to oxidants H_2O_2 and t-BOOH (lethal concentrations) in EG103 isogenic yeast strains. A) co-treated with 8-OASQ and H_2O_2 for 1h; B) pre-treated with 8-OASQ for 1h then challenged with H_2O_2; C) co-treated with 8-OASQ and t-BOOH for 1h; D) pre-treated with 8-OASQ for 1h then challenged with t-BOOH. Percentage survival is expressed relative to untreated control cultures (100%). Values shown are the mean of at least three determinations. * $p<0.05$; ** $p<0.01$ indicate significant increased in the survival % and $^{a}p<0.05$; $^{b}p<0.01$ indicate significant decreased in the survival % compared to the oxidant-treated samples (ANOVA, Dunnet's test).

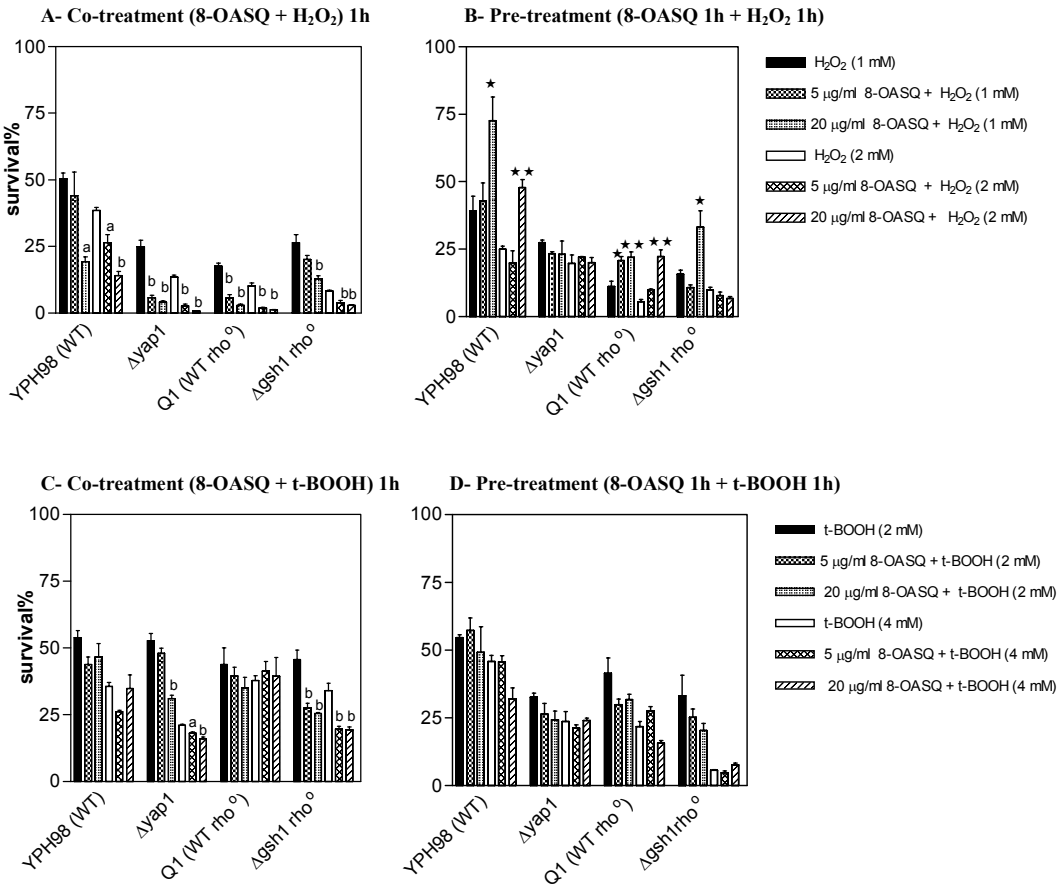

Figure 5. Effect of non-cytotoxic concentrations of 8-OASQ on survival to oxidants H_2O_2 and t-BOOH (lethal concentrations) in YPH98/Q1 isogenic yeast strains. A) co-treated with 8-OASQ and H_2O_2 for 1h; B) pre-treated with 8-OASQ for 1h then challenged with H_2O_2; C) co-treated with 8-OASQ and t-BOOH for 1h; D) pre-treated with 8-OASQ for 1h then challenged with t-BOOH. Percentage survival is expressed relative to untreated control cultures (100%). Values shown are the mean of at least three determinations. * $p<0.05$; ** $p<0.01$ indicate significant increased in the survival % and $^{a}p<0.05$; $^{b}p<0.01$ indicate significant decreased in the survival % compared to the oxidant-treated samples (ANOVA, Dunnet's test).

4. DISCUSSION

Frameshift induction by APO and 8-OASQ in bacteria: The mutagenicity results leave no doubt that both APO and, even more efficiently, its autoxidation product 8-OASQ do induce frameshift mutations (insertion or deletion of one or more base pairs into the DNA) in TA98 and TA97 (Table 2). This mutagenic activity is neutralized in the presence of S9 mix (Table 2). The ability of the oxidized APO form, 8-OASQ, to induce higher levels of frameshift mutations than APO may reside in its more aromatic and planar structure, a feature of quinone compounds [14,36,37]. This presumably favors intercalation into DNA, hence promoting frameshift mutations. In addition, the mutagenesis induced by APO could be the result of some autoxidation during the time-course of the experiments, generating 8-OASQ or other active compounds. The oxidation of APO into more mutagenic products was first suggested by Suter and Matter-Jaeger [21], who also observed induction of frameshift mutations by APO in *S. typhimurium* and related it to the appearance of a dark discoloration in the tester plates (not observed in our experiments), which could be inhibited by anaerobiosis, the S9 mix and by lower than physiological levels of glutathione and/or catalase and superoxide dismutase. The S9 mix could have inhibited the oxidation of APO to 8-OASQ, since it contains enzymes and cofactors such as catalase, glucose-6-phosphatase (G6P), nicotinamide adenine dinucleotide phosphate (NADP) and reduced glutathione (GSH), among others [14,19-21]. In addition, it is probable that the inhibition of 8-OASQ mutagenicity by S9 mix is influenced by its binding to proteins present in S9. The autoxidation products of APO have been reported to bind nonspecifically with proteins, including liver microsomal proteins, forming green conjugates [19].

Mutagenesis of 8-OASQ and antimutagenesis of APO in oxidant sensitive bacterial strains: *E. coli* strains WP2 *uvrA*/pKM101 (IC188) and WP2 *uvrAoxyR*/pKM101 (IC203) carry the *trpE65* ochre mutation and detect a similar spectrum of mutagens as *S. typhimurium* strain TA102 [25,27]. *E. coli* IC188-IC203 has a proven detection ability for mutagens that generate ROS and cause oxidative DNA damage [26] and has been successfully used in detecting such mutagens [27,30]. Higher mutagenic response of strain IC203 than of the IC188 to a substance may be interpreted as an indication of oxidative mutagenesis, since both strains have the same genetic background apart from the OxyR deficiency of IC203 [26,27]. OxyR is a transcriptional regulator of many genes involved in the response to oxidants and strain IC203 was developed to detect oxidant mutagens specifically [26,27]. Since the mutagenicity of 8-OASQ was higher in IC203 than in IC188, we can conclude that 8-OASQ induced oxidative mutagenesis in *E. coli* strains in the absence of metabolic activation, while APO was non-mutagenic under the assay conditions (Table 3). The failure of 8-OASQ to induce mutagenicity in strain TA102 (Table 3) could be ascribed to the repair proficiency of this strain, whereas WP2-derived strains are *uvrA* defective, which impairs excision repair [25,28,29,38]. It is not unusual for the WP2 strains to be more sensitive to some mutagens than the TA102 strain [28,38].

In the presence of the S9 mix, all mutagenic effects of 8-OASQ for these strains are lost and mutagenicity falls back to spontaneous levels (Table 4). In a validation study of strain IC203, Martínez et al. [27] demonstrated that mutagenesis by all the investigated oxidative mutagens, with the exception of the two organic hydroperoxides – t-butyl and cumene hydroperoxide, was inhibited by S9 in the WP2 Mutoxitest. This effect was attributed mainly to the activity of catalase present in the S9 mix. We believe that the

abolition of 8-OASQ oxidative mutagenesis by S9 mix was not due only to the presence of antioxidant factors but also to non-specific binding to proteins.

The possible antimutagenic effects of APO were evaluated by the pre-treatment procedure prior to exposure to the test mutagen. In the absence of metabolic activation, APO strongly and dose-dependently reduced the mutagenicity of H_2O_2 and t-BOOH in strains TA102, IC188, and IC203 (Table 5). The hypothesis that APO protects cells by acting as a free radical scavenger has been raised by various studies [8-11,17,18]. Gassen et al. [18], in cell culture experiments employing rat pheochromocytoma (PC12) cells, demonstrated that APO is a potent cytoprotective agent against oxidative stress induced either by hydrogen peroxide or the neurotoxin 6-hydroxydopamine; it also inhibits iron-induced mitochondrial lipid peroxidation in rat brain [17]. The neuroprotective action of APO in rats, shown using the 6-hydroxydopamine or MPTP models of PD, has been attributed to its iron chelating and radical scavenging properties [9]. Chen et al. [10] demonstrated that APO is a potent hydroxyl radical scavenger which may attenuate iron- and, particularly, dopamine-induced 2,3-dihydroxybenzoic acid (2,3-DHBA) formation. In view of the observed antimutagenic effects, we suggest a protective action of APO against oxidative damage.

In the presence of the S9 mix, the H_2O_2 doses used could not be considered mutagenic (Table 6); this means that APO cannot be evaluated under these conditions. However, the S9 mix, which had no major effect on t-BOOH induced mutagenesis, negatively influenced the antimutagenic effect of APO. In the presence of the S9 mix, APO still offered significant protection against t-BOOH-induced mutagenesis (Table 6), but to a much lower degree than without metabolic activation. We suspect that APO, like its colored sub-product 8-OASQ, binds to proteins in the S9 mix.

Sensitivity to APO and 8-OASQ in WT and S. cerevisiae strains disrupted for key antioxidant genes: The mutant strains showed the same sensitivity to higher doses of APO (200 to 400 μg/ml) (Fig.1A) as seen at lower doses (60 to 100 μg/ml) for 8-OASQ (Fig.1C). Here, the autoxidation derivatives of APO were possibly significant. Sensitivity of yeast strains to the autoxidized form 8-OASQ was clearly dose and strain-dependent (Fig.1C), confirming that at least this and maybe other autoxidation products of APO are more cytotoxic than APO itself. The strains lacking cytosolic forms of catalase and superoxide dismutase were the most sensitive of all single mutants (Fig.1C), indicating an important role of both enzymes in the detoxification of 8-OASQ. The double mutants Δ*ctt1*Δ*sod1* and Δ*sod1*Δ*sod2* were even more sensitive (Fig.1C), indicating a non-epistatic interaction. These responses suggest that 8-OASQ could be involved in the normal redox reactions of quinones and semiquinones, generating both H_2O_2 or $O_2^{\bullet}$ as well as quinones and semiquinone radicals [36,39].

Sensitivity of APO and 8-OASQ by WT and S. cerevisiae strains disrupted for the GSH1 gene and the transcriptional regulator YAP1: These strains have the YPH98 background. The YPH98 WT and the derived Δ*yap1* disruption mutant are respiratory proficient (rho^+) strains; whereas the Q1 WT and the Q2 Δ*gsh1* (γ-glutamylcysteine synthetase) mutant are derived petite strains (Table 1). All strains were somewhat sensitive to higher doses of APO and more so to 8-OASQ (Fig.1B and D). The WT grande and petite strains behaved similarly, while the Δ*gsh1* strain was slightly more sensitive to both products.

We used a yeast mutant that is unable to synthesize the tripeptide glutathione, since several studies have suggested a role for glutathione or thiols in the prevention of both

apomorphine autoxidation and cell damage [20,21]. Glutathione is the most prevalent low molecular weight thiol in the cells and is important for maintaining the intracellular redox-state, acting both as co-factor for enzymes and as antioxidant [40].

Looking at the effect that apomorphine elicited for the glutathione synthesis deficient strain Δ*gsh1* and the strain lacking the oxidative-stress responsive transcription factor Yap1p (Fig.1B), the sensitivity of the latter to 8-OASQ overwhelms by far those of the former (Fig.1D). Yap1p has been shown to regulate a broad set of genes in response to oxi-stress, including most of those involved in glutathione synthesis [41-44], and Δ*yap1* mutants are very sensitive to oxidative stress [34,45-48]. The strong effect of the absence of the Yap1p transcription factor indeed indicates the participation of the cellular antioxidation system in 8-OASQ detoxification, underscoring literature data about the participation of reactive oxygen species formed by a quinone-derivative of APO [1,11,20,36,39]. Yap1p is known to regulate at least two efflux pumps, members of both transporter superfamilies involved in multiple drug resistance (MDR), i.e. the major-facilitator-superfamily transporter Flr1p, which is located in the cytoplasmic membrane, and the ATP-binding-cassette (ABC) transporter (glutathione S-conjugate pump), Ycf1p, which is present in the vacuolar membrane [49,50]. However, before we confer a minor role in this process to the glutathione detoxifying pathways, we must keep in mind that, as the Δ*gsh1* mutant is auxotrophic for glutathione [41,51], it only grows in a medium which is supplemented with glutathione. YPD medium does not need to be supplemented, as it already contains 15-20 mg/L of the tripeptide (M. Brendel personal communication). In the Δ*gsh1* mutant the expression of the other gene products of the glutathione system, such as glutathion S-transferases, glutaredoxins, glutathione reductase, Ycf1p (gsh-dependent vacuolar ABC-transporter),

probably remains unaffected, which is not the case for the Δ*yap1* mutant, since Yap1p is a regulator for most of them [50,52].

Effects of APO and 8-OASQ in hydroperoxide stressed yeast: For assessing pro- or anti-oxidant effects, the cells were treated with non-cytotoxic concentrations of either APO or 8-OASQ together with or prior to exposure to a deleterious concentration of H_2O_2 or t-BOOH. A statistically significant protection by either APO or 8-OASQ was considered as an antioxidant effect and a significant deleterious effect on cellular viability as a pro-oxidant effect (Figs. 2-5).

The effects of APO and 8-OASQ are difficult to analyze individually for every strain, since the experiments were performed under growth conditions with relatively short term exposure to the drugs (1-2h). The mutants studied have deletions in genes that affect their intracellular redox status [35,47,53-56], and possibly influence the redox properties of the growth media. Furthermore, some mutations lengthen the cell replication time, which is normally 1.5 hours, to 2 hours or more, when under stress (Maris and Picada, unpublished observations). There are many variables to consider, in addition to the lack of stability of APO under the tested conditions. Therefore we evaluated the drugs based on the overall pattern of effects elicited for each set of strains and each experimental condition.

APO significantly enhanced the survival of the yeast cells in the pre- and co-treatment procedures with H_2O_2 and t-BOOH (Figs.2 and 3), which points to the antioxidant effects of APO against these hydroperoxides. In contrast, the non-cytotoxic doses of 8-OASQ increased the deleterious effects in the co-treatment experiments with both oxidative agents (Figs.4A,C and 5A,C) in particular when higher oxidant concentration was used (Fig.5A and C), indicating pro-oxidant activities. Pre-treatments with 8-OASQ seemed to

elicit a significant protection to subsequent oxidant challenge for most strains (Figs.4B,D and 5B). These results suggest that during the pre-treatment, 8-OASQ induced cellular anti-oxidant defenses, probably by promoting cross-adaptive stress responses. An adaptive response to oxi-stress permits cells pretreated with sub-lethal concentrations of an oxidant to induce a protective response that allows them to survive a challenge with higher and usually lethal concentrations of the oxidant. Pre-treatment with one kind of stressing agent may also induce cross-resistance against another type [52-54,57].

Final remarks: Our results corroborate previous reports of varying anti- or pro-oxidant actions of APO, depending on its oxidation state. APO can act as an antioxidant, possibly depending on whether the redox-state of its surroundings favors its catechol moiety, avoiding the transition to quinone or semi-quinone. However, high concentrations of APO seem to increase accumulation of sub-products which can either be deleterious or, when in adequate (low) concentrations, can increase the cellular antioxidant capacity by induction of adaptive systems.

The significant mutagenic (frameshift induction) action of both APO and 8-OASQ was noteworthy. This was first described by Suter and Matter-Jaeger [21], but partially dismissed by the pharmaceutical industry. The higher frameshift responses to 8-OASQ reinforce again that the products of APO autoxidation are more deleterious than APO itself, but this does not exclude a significant role for APO in the process. Clearly, these results warrant further studies to characterize the modulatory effects of APO and its oxidized products on the cellular redox-state and their genotoxic activities.

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III – CAPÍTULO II

Differential neurobehavioral deficits induced by apomorphine and its oxidation product, 8-oxo-apomorphine-semiquinone, in rats

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Differential neurobehavioral deficits induced by apomorphine and its oxidation product, 8-oxo-apomorphine-semiquinone, in rats

Jaqueline N. Picada [a], Nadja Schröder [b], Ivan Izquierdo [c], João Antônio Pêgas Henriques [a,d], Rafael Roesler [a,d,e,*]

[a] *GENOTOX-Genotoxicity Laboratory, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), 91501-970 Pôrto Alegre, RS, Brazil*
[b] *Departamento de Ciências Fisiológicas, Faculdade de Biociências, PUCRS, 90619-900 Pôrto Alegre, RS, Brazil*
[c] *Centro de Memória, Departamento de Bioquímica, ICBS, UFRGS, 90035-003 Pôrto Alegre, RS, Brazil*
[d] *Departamento de Biofísica, IB, UFRGS, 91501-970 Pôrto Alegre, RS, Brazil*
[e] *Department of Medicine, UNESC, 88806-000 Criciúma, SC, Brazil*

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Abstract

Apomorphine is a potent dopamine receptor agonist, which has been used in the therapy of Parkinson's disease. It has been proposed that apomorphine and other dopamine receptor agonists might induce neurotoxicity mediated by their quinone and semiquinone oxidation derivatives. The aim of the present study was to evaluate the possible neurobehavioral effects of apomorphine and its oxidation derivative, 8-oxo-apomorphine-semiquinone (8-OASQ). Adult female Wistar rats were treated with a systemic injection of apomorphine (0.05 or 0.5 mg/kg) or 8-OASQ (0.05 or 0.5 mg/kg) 20 min before behavioral testing. Apomorphine and 8-OASQ induced differential impairing effects on short- and long-term retention of an inhibitory avoidance task. Apomorphine, but not 8-OASQ, dose-dependently impaired habituation to a novel environment. The memory-impairing effects could not be attributed to reduced nociception or other nonspecific behavioral alterations, since neither apomorphine nor 8-OASQ affected footshock reactivity or behavior during exploration of an open field. The results suggest that oxidation products of dopamine or dopamine receptor agonists might induce cognitive deficits.

Keywords: Apomorphine; Dopamine; Oxidative stress; Parkinson's disease; Behavior; Memory

1. Introduction

The primary pathology characterizing Parkinson's disease is a selective degeneration of dopaminergic neurons in the substantia nigra, *pars compacta*, which project mainly to the striatum (Hirsch et al., 1988). It has been proposed that oxidative stress plays a pivotal role in the neurodegenerative damage associated with Parkinson's disease (Götz et al., 1990; Ben-Shachar et al., 1991; Fahn and Cohen, 1992; Youdim et al., 1994; Ebadi et al., 1996; Jenner, 1998). For instance, reduced glutathione levels (Sian et al., 1994), increased lipid peroxidation in substantia nigra (Dexter et al., 1994), and oxidative DNA damage (Spencer et al., 1994; Dragunow et al., 1997) have been suggested to be involved in the neurodegenerative mechanisms underlying Parkinson's disease.

The dopamine receptor agonist apomorphine has been used for the therapy of Parkinson's disease. Apomorphine is a potent dopamine D_1 and D_2 receptor agonists that promptly enters the brain and accumulates in the striatum (Bianchi and Landi, 1985). Although dopaminergic therapies have been suggested to enhance the progression of Parkinson's disease by producing reactive oxygen species and inducing cytotoxicity (Grandas and Obeso, 1989; Bindolli et al., 1992; Jenner and Brin, 1998; El-Bachá et al., 2001) and apomorphine at high concentration is toxic to cultured neurons (Spencer et al., 1994; Pardo et al., 1995; El-Bachá et al., 2001), there is no evidence for in vivo neurotoxicity of apomorphine. In fact, a growing body of evidence has shown a neuroprotective activity of apomorphine (Gassen et al., 1996, 1998; Grunblatt et al., 2001a,b). Apomorphine displays antiparkinsonian properties similar to those of L-DOPA and has been shown to be useful for treating Parkinson's disease patients, especially in the late

* Corresponding author. GENOTOX-Genotoxicity Laboratory, Sala 112, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS) (Campus do Vale), Av. Bento Gonçalves, 9500, 91501-970 Pôrto Alegre, RS, Brazil. Tel.: +55-51-3316-6069; fax: +55-51-3316-6084.
E-mail address: rroesler@dna.cbiot.ufrgs.br (R. Roesler).

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stages of the disease (Di Chiara and Gessa, 1978; Corboy et al., 1995; Pzedborski et al., 1995).

Increasing evidence suggests that some of the effects of dopamine and dopamine receptor agonists might be mediated by their quinone and semiquinone oxidation derivatives (Graham, 1978; Bindolli et al., 1992; Smythies, 1997; Segura-Aguillar et al., 1998; El-Bachá et al., 2001). Dopamine oxidation derivatives such as dopamine *o*-quinone and *o*-semiquinone occur in the normal brain (Costa et al., 1992), and dopamine neurotoxicity has been suggested to be mediated by its quinone derivatives acting on NMDA glutamate receptors (see Smythies, 1997 for a review). Dopamine, apomorphine, and L-DOPA easily autoxidize, producing quinone and semiquinone derivatives that may lead to the formation of toxic products and superoxide radicals (Graham, 1978; Bindolli et al., 1992; Segura-Aguillar et al., 1998; El-Bachá et al., 2001), and the toxic effects of apomorphine to cultured neurons have been shown to correlate to its autoxidation (El-Bachá et al., 2001). Recently, we described the isolation of an apomorphine autoxidation semiquinone derivative, 8-oxo-apomorphine-semiquinone (8-OASQ), and demonstrated for the first time its mutagenic activity in vitro (Khromov-Borisov et al., 2000).

Given the neuroprotective properties and clinical relevance of apomorphine in the therapy of Parkinson's disease, and the possibility that autoxidation derivatives of apomorphine are involved in mediating its neurobiological effects, it is important to investigate the neurobehavioral and possible neurotoxic properties of apomorphine and its oxidation derivatives in vivo. In the present study, we evaluated the effects of the systemic administration of apomorphine and its autoxidation derivative, 8-OASQ, on aversive memory, habituation, and open field behavior in rats.

2. Materials and methods

2.1. Animals

We have obtained 182 adult female Wistar rats (170–320 g) from our breeding colony. They were housed five to a cage with food and water available ad libitum, and were maintained on a 12-h light/dark cycle (lights on at 07:00 h). All behavioral procedures were conducted between 10:00 and 16:00 h. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

2.2. Drugs and pharmacological procedures

Apomorphine HCl (Sigma) was dissolved in saline with 10% dimethyl sulfoxide (DMSO). 8-OASQ was isolated as a black water-insoluble precipitate after the incubation of apomorphine HCl (5 mg/ml) for 2 days as previously described (Khromov-Borisov et al., 2000). Isolated 8-OASQ was then dissolved in saline with 10% DMSO. Twenty minutes prior to the behavioral procedures, animals were given an intraperitoneal (i.p.) injection of vehicle (10% DMSO in saline), apomorphine (0.05 or 0.5 mg/kg), or 8-OASQ (0.05 or 0.5 mg/kg), in a volume of 1.0 ml/kg body weight. The doses were chosen on the basis of previous reports on the behavioral effects of apomorphine in rats (Doyle and Regan, 1993; Doyle et al., 1996). All solutions were prepared immediately before injections.

2.3. Behavioral procedures

2.3.1. Inhibitory avoidance

Inhibitory avoidance in rodents is a widely used animal model of aversive learning and memory. The step-down inhibitory avoidance apparatus and procedures were described in previous reports (Izquierdo et al., 1997; Roesler et al., 1999, 2000). The inhibitory avoidance box was a $50 \times 25 \times 25$ cm acrylic box whose floor consisted of parallel stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm-wide, 2.5-cm-high platform was placed on the floor of the box against the left wall. Animals were placed on the platform and their latency to step-down on the grid with all four paws was recorded with an automated device. In training sessions, immediately after stepping down on the grid, the animals were given a 0.6-mA, 1.0-s footshock. In retention test sessions, carried out 1.5 h (short-term retention) or 48 h (long-term retention) after training, no footshock was given and the step-down latency (maximum 180 s) was used as a measure of retention.

2.3.2. Footshock reactivity

The footshock sensitivity test was carried out in the same apparatus used for inhibitory avoidance, as described in previous reports (Roesler et al., 1999, 2000). A modified version of the "up-and-down" method (Crocker and Russell, 1984) was used to determine the nociceptive thresholds. The platform was removed and each animal was placed on the grid and allowed a 1-min habituation period prior to the start of a series of footshocks (0.5 s) delivered at 10-s intervals. Shock intensities ranged from 0.1 to 0.6 mA in 0.1-mA increments. The adjustments in shock intensity were made in accordance to each animal's response. Shock intensity was raised by 1 unit when no response occurred and lowered by 1 unit when a response was made. A "flinch" response was defined as withdrawal of one paw from the grid floor, and a "jump" response was defined as a rapid withdrawal of three or four paws. Two measurements of the "flinch" threshold were made and then two measures of the "jump" threshold were made. For each animal, the mean of the two scores for the flinch and jump thresholds was calculated.

2.3.3. Open field behavior

Animals used in the open field and habituation experiments had been previously trained in inhibitory avoidance.

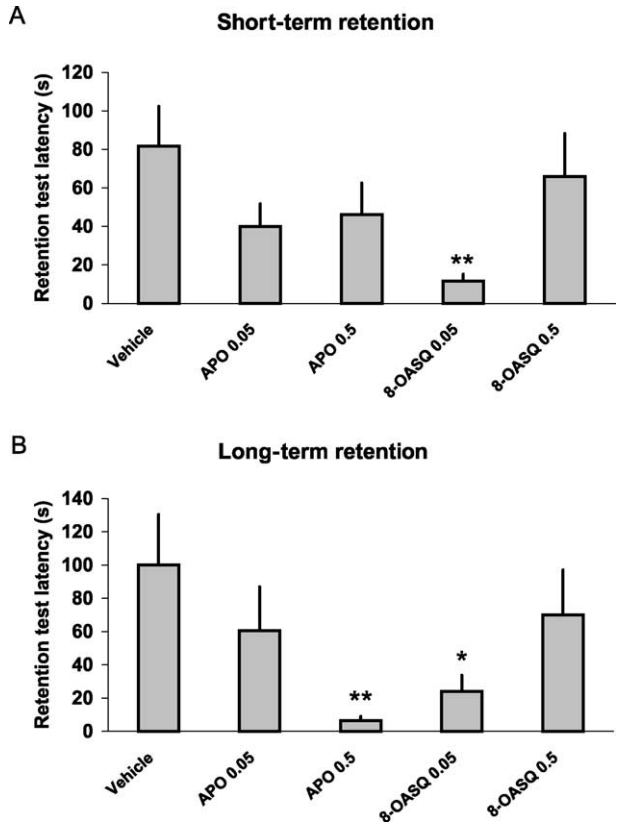

Fig. 1. Effect of pretraining administration of apomorphine (APO) (0.05 or 0.5 mg/kg) and 8-oxo-apomorphine-semiquinone (8-OASQ) (0.05 or 0.5 mg/kg) on (A) short- (1.5 h after training) and (B) long-term (48 h after training) retention of inhibitory avoidance. Animals were given an i.p. injection of vehicle, apomorphine, or 8-OASQ 20 min prior to training. Data are means ± S.E. retention test latencies (s). $N = 8-10$ animals per group; * $P < 0.05$ and ** $P < 0.01$ compared to the vehicle group.

The open field was 50 × 25 cm, surrounded by 50-cm high walls, made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal squares by black lines. One week after the end of the inhibitory avoidance experiment, rats were put in the open field, placed on its left rear quadrant, and left to freely explore the arena for 5 min. Crossing of the black lines, rearings performed, latency to start locomotion, and the number of fecal pellets produced during exploration were counted and used as measures of locomotion, exploration, motivation, and anxiety (Roesler et al., 2000).

2.3.4. Habituation

Long-term retention of habituation to a novel environment can be considered a nonassociative, nonaversive type of learning, which can be measured by the decrease in the exploratory activity as assessed by the number of rearings performed in a test session carried out 24 h after the first exploration session (Vianna et al., 2000). The animals used in the evaluation of open field behavior were reexposed (test session) for 5 min to the open field 24 h after the first exposure (training session), and the number of rearings performed was recorded. The decrease in the number of rearings performed between the first and the second exploration sessions was taken as a measure of habituation.

2.4. Statistics

Data are expressed as mean ± S.E. Differences among groups were analysed with a one-way analysis of variance (ANOVA) followed by an LSD post-hoc test when necessary. Comparisons between the number of rearings performed in training and test sessions within the same group in the habituation experiment were done with a paired *t*-test. In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effects of pretraining administration of apomorphine or 8-OASQ on short- and long-term retention of inhibitory avoidance

Short- and long-term retention of inhibitory avoidance were evaluated in different animals. There were no significant differences among groups in training performance (short-term retention experiment, $P = 0.44$; overall mean ± S.E. training latency = 12.83 ± 1.47; long-term retention experiment; $P = 2.47$; overall mean training latency = 8.59 ± 1.18). Fig. 1A shows the short-term (1.5 h) retention of inhibitory avoidance in rats given an i.p. injection of vehicle, apomorphine (0.05 or 0.5 mg/kg), or 8-OASQ (0.05 or 0.5 mg/kg) 20 min prior to training. 8-OASQ at the dose of 0.05 mg/kg, but not apomorphine at either dose used or 8-

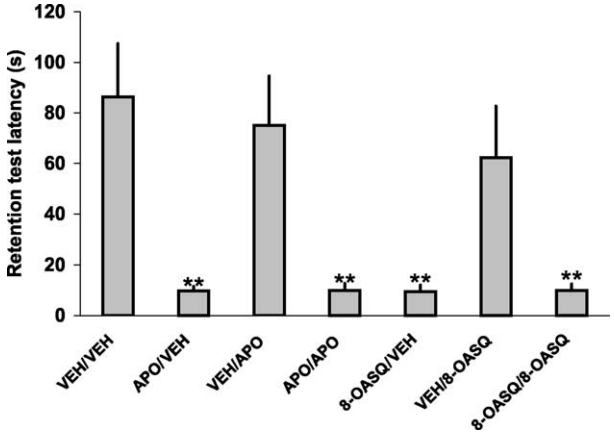

Fig. 2. Effect of combined pretraining and pretest administration of apomorphine (APO) (0.5 mg/kg) and 8-oxo-apomorphine-semiquinone (8-OASQ) (0.05 mg/kg) on long-term (48 h after training) retention of inhibitory avoidance. Animals were given an i.p. injection of vehicle (VEH), apomorphine, or 8-OASQ 20 min prior to training and 20 min prior to test. Data are means ± S.E. retention test latencies (s). $N = 10$ animals per group; ** $P < 0.01$ compared to the VEH/VEH group.

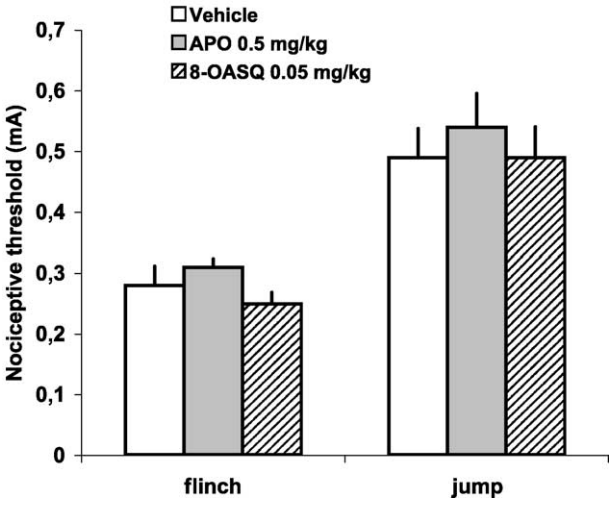

Fig. 3. Effect of apomorphine (APO) (0.5 mg/kg) and 8-oxo-apomorphine-semiquinone (8-OASQ) (0.05 mg/kg) on reactivity to the footshock. Animals were given an i.p. injection of vehicle, apomorphine, or 8-OASQ 20 min prior to testing nociceptive thresholds. Data are means ± S.E. nociceptive thresholds (mA). $N = 7$ animals per group. There were no significant differences between groups.

OASQ at the dose of 0.5 mg/kg, impaired short-term inhibitory avoidance retention. The effects of apomorphine and 8-OASQ on long-term (48 h) retention is shown in Fig. 1B. Apomorphine at the dose of 0.5 mg/kg and 8-OASQ at the dose of 0.05 mg/kg, but not apomorphine at the dose of 0.05 mg/kg or 8-OASQ at the dose of 0.5 mg/kg, impaired retention test performance. When retrained and retested drug-free 1 week after the first training, animals previously treated with 0.5 mg/kg apomorphine or 0.05 mg/kg 8-OASQ and tested for 48-h retention showed normal inhibitory avoidance learning ability (mean ± S.E. overall drug-free training latency = 14.71 ± 1.97, $P = 0.93$; mean ± S.E. 48-h retention test latency = 159.53 ± 20.48 in the animals previously treated with apomorphine 0.5 mg/kg, and 139.85 ± 26.30 in animals previously treated with 8-OASQ 0.05 mg/kg), indicating that the impairing effects of apomorphine and 8-OASQ could not be attributed to an irreversible impairing effect or permanent neuronal damage.

3.2. Effects of combined pretraining and pretest administration of apomorphine and 8-OASQ on long-term retention of inhibitory avoidance

In order to evaluate the possible contribution of state dependency on the memory-impairing effects of apomorphine and 8-OASQ, as well as the possible effects of apomorphine and 8-OASQ on memory retrieval, we verified the effects of combined pretraining and pretest injections of memory-impairing doses of apomorphine and 8-OASQ on inhibitory avoidance retention. Thus, the resulting pretraining/pretest treatment groups were vehicle/vehicle, apomorphine/vehicle, vehicle/apomorphine, apomorphine/apmorphine, 8-OASQ/vehicle, vehicle/8-OASQ, and 8-

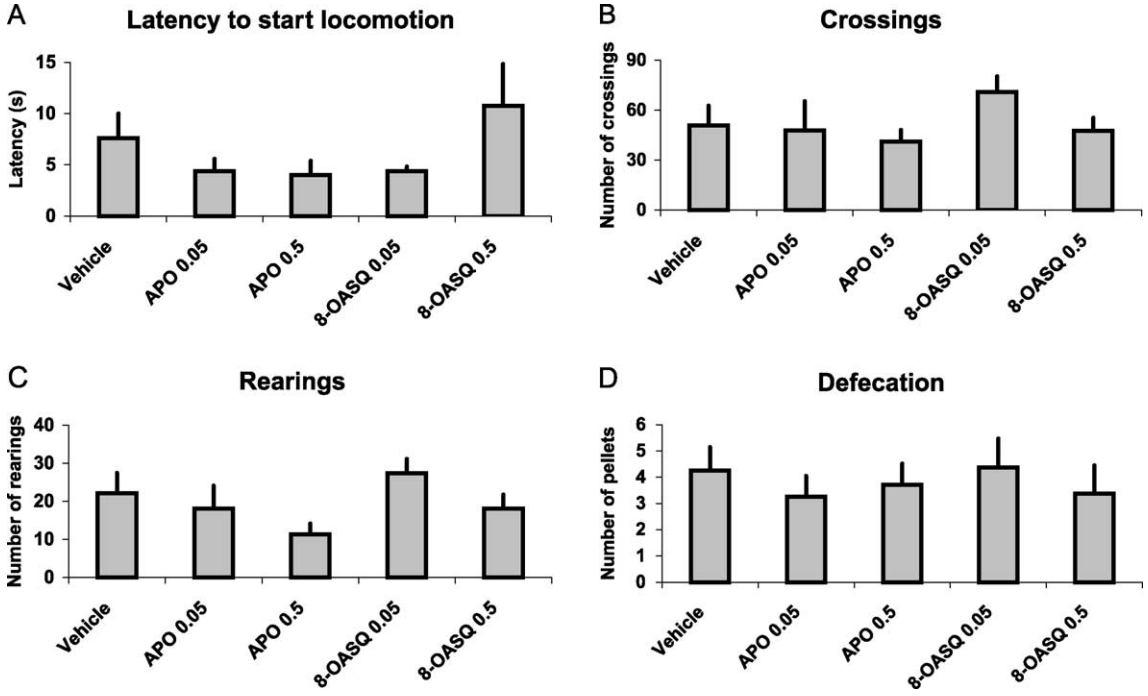

Fig. 4. Effect of pretraining administration of apomorphine (APO) (0.05 or 0.5 mg/kg) and 8-oxo-apomorphine-semiquinone (8-OASQ) (0.05 or 0.5 mg/kg) on the (A) latency to start locomotion, (B) number of crossings performed, (C) number of rearings performed, and (D) number of fecal pellets produced during a 5-min exploration of an open field. Animals received an i.p. injection of vehicle, apomorphine, or 8-OASQ 20 min prior to being exposed to the open field. Data are expressed as means ± S.E. $N = 7–8$ animals per group. There were no significant differences between groups.

OASQ/8-OASQ. There were no significant differences among groups in training performance ($P = 0.89$; overall mean ± S.E. training latency = 10.01 ± 0.70). Retention test latencies are shown in Fig. 2. Combined pretraining and pretest treatments with either apomorphine or 8-OASQ failed to reverse the retention deficits, indicating that the impairing effects do not involve state dependency. Moreover, pretest administration of apomorphine or 8-OASQ did not affect retrieval, as shown by the normal retention in the vehicle/apomorphine and vehicle/8-OASQ groups, showing that those drugs impaired the formation, but not the expression of inhibitory avoidance memory.

3.3. Lack of effect of apomorphine and 8-OASQ on reactivity to the footshock

In order to verify whether the impairing effects of apomorphine and 8-OASQ on memory for inhibitory avoidance could be due to a reduction in nociception, we evaluated the effects of apomorphine and 8-OASQ at the doses shown to be effective in inhibitory avoidance on footshock sensitivity. Reactivity to the footshock assessed by flinch and jump thresholds was not affected by apomorphine or 8-OASQ (Fig. 3), indicating that the memory-impairing effects of pretraining injections of those drugs were not due to reduced nociceptive response.

3.4. Lack of effect of apomorphine and 8-OASQ on open field behavior

Behavior during a 5-min exploration of an open field in rats given apomorphine or 8-OASQ 20 min pretraining is shown in Fig. 4. One animal treated with apomorphine 0.5 mg/kg showed stereotyped behavior (rapid, repetitive forelimb and head movements) during open field exploration and was excluded from the experiment. There were no significant differences among groups in the number of crossings or rearings performed, latency to start locomotion, or number of fecal pellets produced. These findings suggest that apomorphine and 8-OASQ did not affect locomotion, exploration, motivation, or anxiety.

3.5. Effects of apomorphine and 8-OASQ on habituation to an open field

The habituation of rearings in rats submitted to a training and a test session of exploration in the open field is shown in Fig. 5. The group treated with 0.5 mg/kg of apomorphine 20 min pretraining showed a significantly higher number of rearings during test compared to the vehicle-treated group. In addition, all groups showed a significant decrease in the number of rearings in the test session in comparison with the training session, except the group given apomorphine at 0.5 mg/kg (comparisons between training and test sessions within each group by paired *t*-tests, $P = 0.27$ in the apomorphine 0.5 mg/kg-treated group). These results show that apomorphine, but not 8-OASQ, dose-dependently impaired long-term habituation to a novel environment.

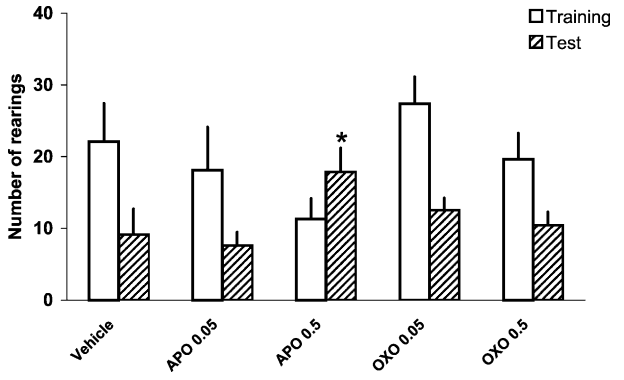

Fig. 5. Effect of pretraining administration of apomorphine (APO) (0.05 or 0.5 mg/kg) and 8-oxo-apomorphine-semiquinone (8-OASQ) (0.05 or 0.5 mg/kg) on habituation to an open field. Animals received an i.p. injection of vehicle, apomorphine, or 8-OASQ 20 min prior to training. Data are means ± S.E. number of rearings performed. $N = 7–8$ animals per group; * $P < 0.05$ compared to the vehicle group.

4. Discussion

The results of the present study show that systemic pretraining administration of the dopamine receptor agonist apomorphine and the oxidation product of apomorphine, 8-OASQ, induced differential impairing effects on short- and long-term retention of a step-down inhibitory avoidance task in rats, whereas apomorphine, but not 8-OASQ, impaired habituation. The impairing effects of apomorphine and 8-OASQ on inhibitory avoidance were not due to state dependency. Neither drug affected inhibitory avoidance retrieval, footshock reactivity, or behavior during exploration of an open field.

Apomorphine is a classical nonselective dopamine receptor agonist and antiparkinsonian therapeutic agent (Di Chiara and Gessa, 1978; Bianchi and Landi, 1985; Corboy et al., 1995; Pzedborski et al., 1995). Although the main clinical symptoms of Parkinson's disease are motor disturbances (Duvoisin, 1991), animal studies have suggested that Parkinson's disease also involves learning and memory disabilities (Roeltgen and Schneider, 1994; Fernandez Ruiz et al., 1995; Brown et al., 1997; Da Cunha et al., 2001), and Parkinson's disease patients show cognitive deficits, including memory impairments (Dubois and Pillon, 1997; Goldman et al., 1998). Thus, it is important to characterize the cognitive effects of dopaminergic drugs used in Parkinson's disease therapy and their metabolites. Some of the findings described in the present report, namely the impairing effect of apomorphine on inhibitory avoidance and the lack of effect on open field exploration, are consistent with previous studies (Davies et al., 1974; Ichihara et al., 1988; Doyle and Regan, 1993; Doyle et al., 1996). The aversive memory-impairing effect of apomorphine reported in the present

study suggests that activation of central dopaminergic receptors by apomorphine dose-dependently impair the formation, but not the expression of long-term inhibitory avoidance memory, and retention of the nonaversive, non-associative habituation task, without affecting short-term retention, locomotion, motivation, anxiety, or nociception. By contrast, the oxidation derivative of apomorphine, 8-OASQ, at the lower but not at the higher dose used, impaired both short- and long-term retention of inhibitory avoidance without affecting habituation, suggesting that apomorphine and its oxidized product 8-OASQ display differential pharmacological actions. Although several drugs have been shown to affect memory in lower doses while higher doses are ineffective, further studies are necessary to clarify the dose–response pattern of the mnemonic effect of 8-OASQ and the mechanisms underlying it.

Although in vitro studies show that apomorphine can be toxic to cultured neurons, inducing cytotoxicity and DNA damage through oxidative stress (Spencer et al., 1994; Pardo et al., 1995; El-Bachá et al., 2001), evidence for in vivo neurotoxicity of apomorphine remains lacking, and apomorphine has been proposed as a potential neuroprotective in vivo (Gassen et al., 1998; Chen et al., 2001; Fornai et al., 2001). Increasing evidence suggests that apomorphine displays a neuroprotective activity mediated by its free radical scavenging properties (Mytilineou et al., 1993; Gassen et al., 1996, 1998; Chen et al., 2001; Grunblatt et al., 2001a,b). Both *R*-apomorphine and its isomer, *S*-apomorphine, which is not a dopamine receptor agonist, have been shown to exert neuroprotective effects in the *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice model of Parkinson's disease (Grunblatt et al., 2001a).

Recent studies suggest that some of the neurobiological effects of dopamine and dopamine receptor agonists such as apomorphine might be mediated by their oxidation products, and the effects of dopamine receptor agonists quinone and semiquinone derivatives might be importantly involved in the neurodegeneration associated with Parkinson's disease (Graham, 1978; Bindolli et al., 1992; Smythies, 1997; Segura-Aguillar et al., 1998; El-Bachá et al., 2001). Although the mechanisms underlying the effects of dopamine receptor agonists quinone and semiquinone derivatives are still unclear, there is evidence indicating that they stimulate NMDA receptor-mediated excitotoxicity by acting on NMDA receptors (Smythies, 1997), bind irreversibly to intracellular proteins forming conjugates (El-Bachá et al., 2001), and induce the formation of reactive oxygen species, initiating intracellular oxidative stress (Graham, 1978; Bindolli et al., 1992; Segura-Aguillar et al., 1998; El-Bachá et al., 2001).

To our knowledge, the present study is the first to evaluate the neurobehavioral activity of an isolated apomorphine autoxidation derivative. Our findings show that a single, systemic administration of 8-OASQ, at doses comparable to memory-impairing doses of apomorphine, is capable of inducing impairments on retention of an aversive memory task without affecting other behavioral parameters. The effects were not due to a long-lasting impairing effect or permanent neuronal damage since rats treated with either apomorphine or 8-OASQ were able to learn normally when retrained drug-free 1 week later. Moreover, 8-OASQ affected memory at the lowest, but not at the highest dose used.

The present findings suggest that oxidation derivatives of apomorphine and possibly those of dopamine and L-DOPA clinically used in the therapy of Parkinson's disease might display pharmacological actions on mechanisms involved in emotional learning and memory processes and induce cognitive deficits. An interesting possibility is that 8-OASQ might have antioxidant properties and thus exert neuroprotective effects. Further experiments are currently being carried out in our laboratory in order to investigate this possibility, as well as to clarify the cellular and molecular mechanisms underlying the neurobehavioral effects of 8-OASQ.

Acknowledgements

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IV – CAPÍTULO III

An oxidized form of apomorphine fails to induce stereotypy

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Letter to the Editor

An oxidized form of apomorphine fails to induce stereotypy☆

To the Editors,

Stereotyped behaviors are features of psychiatric disorders such as schizophrenia and obsessive–compulsive disorder (Ridley, 1994). Stereotypy can be induced with dopamine agonists such as amphetamine and apomorphine, and apomorphine-induced stereotypy in rodents has been considered an animal model of psychosis. Apomorphine is a potent D_1 and D_2 dopamine receptor agonist that displays neuroprotective actions (Grunblatt et al., 2001) and antiparkinsonian properties similar to those of L-DOPA, and has been increasingly used in the therapy of Parkinson's disease (PD) (Corboy et al., 1995).

It has been suggested that some of the neurobiological effects of apomorphine and other dopamine receptor agonists are mediated by their oxidation derivatives. Oxidation derivatives of dopamine occur in the normal brain, and dopamine, apomorphine, and L-DOPA easily autoxidize, producing quinone and semiquinone derivatives that may lead to the formation of superoxide radicals and induce neurotoxicity (Bindolli et al., 1992; Smythies, 1997; El-Bachá et al., 2001). It is possible that oxidation reactions similar to those involved in the production of oxidized derivatives of dopamine agonists play a role in the pathogenesis of neuropsychiatric disorders (see Smythies, 1997, for a review).

The mechanisms underlying the effects of oxidation derivatives of dopamine agonists remain unclear. The investigation of the neurobehavioral actions of oxidation derivatives of apomorphine and other dopamine agonists might help elucidating the mechanisms involved in the effects of apomorphine in PD patients as well as the cellular and molecular processes involved in psychiatric disorders and in the degeneration of dopaminergic neurons associated with PD. We recently isolated for the first time an apomorphine autoxidation derivative, 8-oxo-apomorphine-semiquinone (8-OASQ) (Khromov-Borisov et al., 2000) and showed that it induces neurobehavioral deficits in rat models of learning and memory (Picada et al., 2002). Here we describe a first attempt to investigate the mechanisms underlying the central effects of an isolated dopamine agonist oxidation derivative by evaluating whether administration of 8-OASQ is able to induce stereotypy in mice. A failure of 8-OASQ to induce stereotypy would indicate that the oxidation of apomorphine causes a loss of affinity for dopamine receptors.

Fifty-four adult male CF-1 mice were housed with food and water available ad libitum, and were maintained on a 12-h light/dark cycle. Apomorphine HCl (Sigma) was dissolved in saline with 10% dimethyl sulfoxide (DMSO). 8-OASQ was isolated as a black water-insoluble precipitate as previously described (Khromov-Borisov et al., 2000; Picada et al., 2002). Isolated 8-OASQ was then dissolved in saline with 10% DMSO. All solutions were prepared immediately prior to administration. Animals were given an intraperitoneal (i.p.) injection of vehicle (10% DMSO in saline), apomorphine (2.5, 5.0 or 10.0 mg/kg) or 8-OASQ (2.5, 5.0 or 10.0 mg/kg). After drug injection, mice were placed three per cage and were evaluated for stereotyped behavior for 1 min at 10-min intervals during 1 h. Stereotypy was defined as rapid, repetitive head and forelimb movements (Battisti et al., 2000). Data are expressed as mean ± SE percentage of time showing stereotypy. Differences among groups were analyzed with a one-way analysis of variance (ANOVA) followed by a LSD post-hoc test when

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necessary; $p < 0.05$ was considered to indicate statistical significance.

Systemic administration of apomorphine dose-dependently induced stereotypy in mice, whereas animals treated with 8-OASQ at any of the doses tested failed to show stereotypy. The mean ± SE percentage of time showing stereotyped behavior was 9.27 ± 4.04 in the group treated with 2.5 mg/kg apomorphine; 25.93 ± 9.66 in the group treated with 5.0 mg/kg apomorphine; and 55.56 ± 11.78 in animals treated with 10.0 mg/kg apomorphine. Animals treated with vehicle or 8-OASQ at the doses of 2.5, 5.0 or 10.0 mg/kg showed no stereotypy. An ANOVA followed by LSD post-hoc testes showed significant differences between the groups treated with either vehicle or 8-OASQ and the groups treated with apomorphine at the doses of 5.0 or 10.0 mg/kg ($p < 0.01$ in all comparisons).

Given that increasing evidence suggests that some of the neuronal effects of dopamine, L-DOPA, and apomorphine are mediated by their oxidation derivatives (Bindolli et al., 1997; Smythies, 1997; El-Bachá et al., 2001), we propose that the investigation of the neurobiological actions of isolated oxidation derivatives of apomorphine is clinically relevant and might help to elucidate the mechanisms involved in oxidative stress-mediated neuronal damage in schizophrenia (Smythies, 1997), neurodegeneration associated with PD, and the effects of dopaminergic therapies in PD patients. The finding reported in the present study that 8-OASQ fails to induce stereotypy in mice suggests that autoxidation of apomorphine causes a loss in its ability to bind dopamine receptors, since stereotypic behaviors are typically associated with an increase in dopaminergic activity induced by either dopamine agonists or drugs indirectly affecting dopaminergic transmission (Ridley, 1994; Battisti et al., 2000). Thus, the neurobiological and possible neurotoxic actions of oxidation derivatives of dopamine agonists might be mediated by mechanisms other than a stimulation of dopamine receptors. Further studies using neurochemical and molecular biology approaches are being carried out in order to clarify the mechanisms of action of apomorphine oxidation derivatives. We are currently investigating the possibility that 8-OASQ displays anti-oxidant and neuroprotective properties.

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Jaqueline N. Picada
João Antônio Pêgas Henriques
Rafael Roesler*
GENOTOX-Genotoxicity Laboratory, Center for Biotechnology, Sala 112, UFRGS, Av Bento Concalves 9500, Porto Alegre, RS 91501-970, Brazil
E-mail address: rroesler@dna.cbiot.ufrgs.br

27 August 2002

* Corresponding author. Tel.: +55-51-3316-6069; fax: +55-51-3316-6084.

V – CAPÍTULO IV

DNA damage in brain cells of mice treated with an oxidized form of apomorphine

(*Molecular Brain Research*, in press)

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Corresponding Author: Dr João A ntonio Pêgas Henriques, PhD Federal University of Rio Grande do Sul

Other Authors: Jaqueline N Picada, Debora G Flores, Cláudio G Zettler, Norma P Marroni, Rafael Roesler

*** Cover Letter**

November 07, 2002

To Dr. J.I. Morgan

Molecular Brain Research

Dear Editor,

Please find enclosed the manuscript entitled *"DNA damage in brain cells of mice treated with an oxidized form of apomorphine"*, by Jaqueline Nascimento Picada, Debora G. Flores, Cláudio G. Zettler, Norma Possa Marroni, Rafael Roesler, João Antonio Pêgas Henriques, which we are submitting for publication in **Molecular Brain Research.**

Look forward to hear from you,

Dr. João Antonio Pêgas Henriques

GENOTOX - Genotoxicity Laboratory, Center for Biotechnology, and Department of Biophysics, Institute of Biosciencies (IB), Federal University of Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 9500, 91501-970, Porto Alegre, RS, Brazil

Fax: +21 51 3316 6084;

E -mail address: pegas@dna.cbiot.ufrgs.br (J.A.P. Henriques)

*** Suggested Reviewers**

November 07, 2002

Dr. J.I. Morgan

Molecular Brain Research

Dear Editor,

We suggest the following researchers as reviewers:

Dr. E. Moustacchi
Institut Curie-Recherche, UMR 218 CNRS, LRC no. 1 CEA, 26 rue d'Ulm, 75248, Paris, France.
(ethel.moustacchi@curie.fr)

Dr. G. Speit
Universitatsklinikum Ulm, Abteilung Humangenetik, D 89070 Ulm, Germany.
(guenter.speit@medizin.uni-ulm.de)

Dr. J. R. Smythies
Department of Neuropsychiatry, Institute of Neurology, Queen Square, London, WCl 3BG, UK
Present address: Brain and Perception Laboratory, Center for Human Information Processing, UCSD, La Jolla, CA 92093-0109, USA.
(smythies@psy.ucsd.edu)

Dr. A. Minn
UMR 7561 CNRS-Université Henri Poincaré-Nancy 1, Laboratoire de Pharmacologie, Faculté de Médecine, B.P. 184, F-54505 Vandoeuvre-lés-Nancy Cedex, France.
(minn@facmed.u-nancy.fr)

Look forward to hear from you,

Dr. João Antonio Pêgas Henriques

GENOTOX - Genotoxicity Laboratory, Center for Biotechnology, and Department of

Biophysics, Institute of Biosciencies (IB), Federal University of Rio Grande do Sul

(UFRGS), Av. Bento Gonçalves, 9500, 91501-970, Porto Alegre, RS, Brazil

Fax: +21 51 3316 6084;

E -mail address: pegas@dna.cbiot.ufrgs.br (J.A.P. Henriques)

Abstract

We investigated whether systemic injection of apomorphine and its oxidation derivative 8-oxo-apomorphine-semiquinone (8-OASQ) can induce DNA damage in mice brain, using the single-cell gel assay. 8-OASQ induced DNA damage in the brains at 1 h and 3 h, but not at 24 h after treatment whereas apomorphine induced a slight increase in brain DNA damage frequency at 3 h after treatment, suggesting that both drugs display a genotoxic activity in brain tissue.

Short Communication

DNA damage in brain cells of mice treated with an oxidized form of apomorphine

Jaqueline Nascimento Picada[a,b], Debora G. Flores[a], Cláudio G. Zettler[c,d], Norma Possa Marroni[e,f], Rafael Roesler[a,g], João Antonio Pêgas Henriques[a]*

[a] *GENOTOX - Genotoxicity Laboratory, Center for Biotechnology, and Department of Biophysics, Institute of Biosciencies (IB), Federal University of Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 9500, 91501-970, Porto Alegre, RS, Brazil*

[b] *Department of Pharmacy, Lutheran University of Brazil (ULBRA) 92420-280 Canoas, RS, Brazil*

[c] *Department of Morphological Sciences, FFFCMPA, 90035-003 Porto Alegre, RS, Brazil*

[d] *Department of Medicine, ULBRA, 92420-280 Canoas, RS, Brazil*

[e] *Department of Physiology, IB, UFRGS, 90035-003 Porto Alegre, RS, Brazil*

[f] *Department of Physiotherapy, ULBRA, 92420-280 Canoas, RS, Brazil*

[g] *Department of Medicine, University of Southern Santa Catarina (UNESC), 88806-000 Criciúma, SC, Brazil*

*Corresponding author. Fax: +21 51 3316 6084; E -mail address: pegas@dna.cbiot.ufrgs.br (J.A.P. Henriques)

Abstract

We investigated whether systemic injection of apomorphine and its oxidation derivative 8-oxo-apomorphine-semiquinone (8-OASQ) can induce DNA damage in mice brain, using the single-cell gel assay. 8-OASQ induced DNA damage in the brains at 1 h and 3 h, but not at 24 h after treatment whereas apomorphine induced a slight increase in brain DNA damage frequency at 3 h after treatment, suggesting that both drugs display a genotoxic activity in brain tissue.

Keywords: Apomorphine; Dopamine; Oxidative stress; DNA damage; Comet assay; Neurotoxicity

Apomorphine (APO; 4*H*-Dibenzo[*de,g*]quinoline-10,11-diol, 5,6,6a,7-tetrahydro-6-methyl-(*R*)) is a potent dopamine D_1 and D_2 receptor agonist which promptly enters the brain and accumulates in the striatum (reviewed by [19]). APO displays anti-Parkinsonian properties similar to those of L-DOPA and has been shown to be useful for treating Parkinson's disease (PD) patients, especially in the late stages of the disease [9,19]. Recently, APO was approved for the treatment of erectile dysfunction [10]. APO can induce neurotoxicity in vitro, and it has been suggested that some of the neurotoxic effects of apomorphine and other dopamine receptor agonists are mediated by their oxidation derivatives [3,11,14]. In addition, oxidation derivatives of dopamine agonists can induce neurotoxicity mediated by *N*-methyl-D-aspartate receptor-dependent excytotoxicity [20].

Recently, we described the isolation, mutagenic activity, and neurobehavioral toxicity of an apomorphine autoxidation semiquinone derivative, 8-oxo-apomorphine-semiquinone (8-OASQ) [13,14,15]. In the present study, the influence of this autoxidation product of APO on DNA damage in the brain was assessed using alkaline comet assay in samples of brain tissue and blood of pre-treated mice with APO and 8-OASQ.

Apomorphine HCl (APO) (Sigma) and 8-oxo-apomorphine-semiquinone (8-OASQ) (isolated as previously described in [13]) were dissolved in saline with 10% dimethyl sulfoxide (DMSO). Male CF-1 mice aged 9 weeks were purchased from the State Foundation for Health Science Research (FEPPS/LACEN-RS, Porto Alegre, Brazil). Animals were housed five to a cage with food and water available *ad libitum*, and were maintained on a 12-h light/dark cycle (lights on at 07:00 h). The mice were acclimated to the laboratory for at least 7 days before the experiments. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of

Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Mice were treated with a single i.p. dose of vehicle, APO (40.0 mg/kg) or 8-OASQ (40.0 mg/kg). A positive control group treated dosed with 40 mg/kg of methyl methane sulfonate (MMS, Sigma, St. Louis, MO) was also carried out. Animals were sacrificed by decapitation 1, 3, or 24 h after treatment, and the forebrain as well as a peripheral blood sample were collected from each animal, according to the method described by [23].

The alkaline comet assay (or single cell gel electrophoresis – SCG) was performed as described by [18] with minor modifications [23]. Each piece of brain was placed in 0.5 mL of cold phosphate-buffered saline (PBS) and minced into fine pieces in order to obtain a cellular suspension. These cells from brain and from the peripheral blood (5-10 μL) were embedded in 95 μL of 0.75% low melting point agarose (Gibco BRL). The mixture (cell/agarose) was added to a fully frosted microscope slide coated with a layer of 300 μL of normal melting agarose (1%) (Gibco BRL). After solidification, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0, with freshly added 1 % Triton X-100 (Sigma) and 10% DMSO) for a minimum of 1 h and a maximum of 2 weeks at 4^{o}C. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH >13) for 20 min, at 4^{o}C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min to electrophorese the DNA. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with ethidium bromide (2 μg/mL) (Sigma) and analyzed using a fluorescence microscope.

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal. Comet image lengths (nuclear region plus tail)

were measured in arbitrary units, with a calibrated scale in the ocular, using a fluorescence microscope equipped with an excitation filter of BP546/12 nm and a barrier filter of 590 nm. One unit was approximately 5 μm at 200 x magnification.

Cells were also scored visually according to tail size into five classes ranging from undamaged (0), to maximally damaged (4), resulting in a single DNA damage score for each animal, and consequently each studied group. Therefore, the damage index (DI) can range from 0 (completely undamaged, 100 cells x 0) to 400 (with maximum damage, 100 x 4) [4,8,17].

The damage frequency (%) was calculated based on number of cells with tail versus those with no tails. The statistical evaluation was performed using the Dunnett test. Values of $p < 0.05$ were considered to indicate statistical significance.

For histopathological evaluation of brain slices, pieces of each brain were fixed with Bouin solution for 12 h and embedded in paraffin before being sectioned and stained with hematoxylin-eosin for verification of cellular death by necrosis. The slides were analyzed using an optical microscope by a trained pathologist (data not shown).

Table 1 shows the effects of APO and 8-OASQ on image length (IL), damage index (DI), and damage frequency (DF), as measured by DNA damage in mice brain tissue, using the comet assay. The brains from mice treated with APO and sacrificed 1 h later showed no significant difference in any of the three parameters used to assess DNA damage in comparison with the vehicle-treated control group, whereas brains from animals treated with 8-OASQ and sacrificed 1 h after treatment showed a significant increase ($p < 0.01$) for all parameters. At the 3 h sampling time both the IL and DI in the group treated with 8-OASQ diminished in comparison to the 1 h treated group, while the DF remained around 60-70%. APO slightly increased the DF (29.5%, *p*

< 0.05) at the 3 h sampling time in comparison to the control group. Those effects were totally abolished in animals sacrificed 24 h after treatment.

We detected no microscopic signs of necrosis in the brains in which DNA damage was observed. Therefore, the DNA damage observed in 8-OASQ-treated mice is not due to the toxic cell death (data not shown).

The results in peripheral blood sample collected of the same mice are presented in Table 2. APO and 8-OASQ did not significantly increase the mean values of IL, DI, and DF.

Fig.1 shows the extent of DNA damage, analyzed using a SCG assay (classes 0-4). In brain, the APO-damaged cells were approximately 20% and 30% class 1 cells, in the groups killed at 1 h and 3 h, respectively. The incidence of other classes was very low at the group sacrificed 1 h after treatment and inexistent in animals killed 3 h after treatment. Classes 1 and 2 were the most frequent among the damaged cells in brains of mice treated with 8-OASQ at 1 h and 3 h sampling times. DNA damage peak was at 1 h, where more than 10% class 4 cells were observed, falling to less than 2% at 3 h. A similar pattern of response was showed for the incidence of class 3 cells in a comparison between groups killed at 1 h and 3 h after treatment with 8-OASQ. Only damaged cells of class 1, with incidence of less than 10%, were found at 24 h groups, showing no significant response of APO and 8-OASQ in relation to vehicle group. In peripheral blood, less than 10% of cells were damaged in all groups (Fig.1), indicating APO and 8-OASQ did not damage blood cell DNA.

The alkaline comet assay is a sensitive procedure used to quantify DNA lesions in mammalian cells. This assay detects DNA strand breaks, alkali-labile sites and incomplete excision repair events in individual cells [23]. Previous studies have shown that the autoxidation products of APO and other catechols (e.g. dopamine) might lead to

deleterious effects on neuronal cells and neural function [3,14], and the cytotoxic effects of APO to cultured neurons have been shown to correlate to its autoxidation products [11]. Our results show a significant increase of DNA damage index (DI) and damage frequency (DF) in mice brain tissue 1 h and 3 h after treatment with 8-OASQ (Table 1), an autoxidation product of APO. Recently, we demonstrated that 8-OASQ displays a higher frameshift mutagenic activity, which stimulates DNA strand breaks, when compared to APO [13]. In addition, it is possible that 8-OASQ induces oxidative DNA damage mediated by its pro-oxidant effects, which were observed in cells of *Saccharomyces cerevisiae* lacking antioxidant-defenses and its oxidative mutagenesis in *Escherichia coli* WP2-derivative strains, in addition to the frameshift in *Salmonella typhimurium* TA98 and TA97 (unpublished results). Probably, OASQ displays these biological effects through usual redox reactions of quinones and semiquinones generating both H_2O_2 or $O_2^{\bullet}$ besides quinones and semiquinone radicals [12]. These reactions could promote an increased formation of $OH^{\bullet}$ radicals, that are able to induce mostly single-strand breaks and various species of oxidized purines and pirimidines [5,7].

In contrast to 8-OASQ, APO did not induce significant DNA damage to brain cells DNA at 1 h after treatment, although it induced a slight increase in the DF at 3 h (Table 1). It is possible that by 3 h after injection APO already had been almost totally metabolized, as the brain APO concentration significantly decreases between 1 h and 2 h after injection [2], and these metabolites contributed to the increased DF observed in brain cells. The APO-induced damages were mostly of the class 1 cell damage (Fig.1), which are considered minimal damages. For 8-OASQ, in animals sacrificed at 3 h after injection classes 3 and 4 damaged cells were found (Fig.1), but in lower levels than in mice killed 1 h after injection, indicating repair of some DNA damage 3 h after drug

administration. However, it is interesting to note that DF remains high at 3 h (64.0±7.4% versus 76.0±6.7% at 1 h) (Table 1). The 24 h sampling time shows a clear DNA repair response as no significant DNA damage for both APO and 8-OASQ could be found (Table 1).

Neither APO nor 8-OASQ induced DNA damage in blood samples (Table 2). Several recent studies showed that the rate of drug-induced genotoxicity detected by the comet assay depends on the tissue or organ analyzed [16,17,24]. APO has been shown to enter the brain and accumulate in the striatum soon after being injected peripherally. Although the mechanisms of action and pharmacokinetics of isolated 8-OASQ has not been described so far, the present results, together with previous evidence from our laboratory that 8-OASQ affects neural function [14], strongly indicates that 8-OASQ also enters the brain when given peripherally. On the other hand, MMS, used as positive control, damaged DNA in both brain and blood samples of the treated mice, showing a non tissue-specific genotoxic activity.

The slight genotoxic effect of APO in the brain is consistent with other studies suggesting that APO displays neurotoxic effects in vitro [11]. However, APO may also play a role in preventing or slowing the rate of neurodegeneration associated with PD by scavenging iron and preventing dopamine-induced hydroxyl radical formation [1,6]. Accordingly, we have observed antimutagenic and antioxidant activities for APO against t-butylhydroperoxide and hydrogen peroxide on oxidative stress- sensitive strains WP2-derivatives *E. coli* and TA102 *S. typhimurium*, and *S. cerevisiae* lacking antioxidant defenses (unpublished results).

Mutagenic and clastogenic effects of APO were shown to be decreased by the presence of metabolic activation S9 mix [21,22]. In addition, cytotoxic effects were prevented by binding of non-specific proteins or by reaction of antioxidant molecules

with reactive species formed during APO oxidation [11,12]. Based on these findings, in the present experiments APO might have been detoxified through similar mechanisms in mice. Interestingly, in spite of predicted detoxification of 8-OASQ by the mice intrinsic metabolic systems, damaged DNA was detected in the brain, indicating a genotoxic effect of APO autoxidation derivatives.

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Legend for figure

Fig.1. Distribution of damage classes (0 – undamaged to 4 – maximum damage) in brain and peripheral blood from mice treated with an i.p. injection of vehicle, apomorphine (APO; 40 mg/kg), or 8-oxo-apomorphine semiquinone (8-OASQ; 40 mg/kg). Frequency was calculated for 100 cells per treatment.

Table 1

Comet assay in brain tissue of mice treated with an i.p. injection of vehicle, apomorphine (APO; 40 mg/kg), or 8-oxo-apomorphine-semiquinone (8-OASQ; 40 mg/kg), and sacrificed 1, 3 or 24 h after injection

Sampling time (h)	Group	Individual results					Mean±SD by group
1	Vehicle	IL	22.7±3.8	25.7±2.3	24.0±3.6	24.3±3.1	24.2±1.2
		DI	13	16	17	18	16.0±2.2
		DF (%)	9	14	15	17	13.8±3.4
	APO	IL	26.9±5.9	25.2±4.8	20.1±4.2	27.3±4.3	24.9±3.3
		DI	30	26	19	33	27.0±6.1
		DF (%)	20	20	16	26	20.5±4.1
	8-OASQ	IL	34.6±10.2	33.2±10.7	35.1±11.7	33.0±9.5	34.0±1.0**
		DI	146	137	167	136	146.5±14.4**
		DF (%)	85	70	72	77	76.0±6.7**
3[d]	Vehicle	IL	20.0±0.9	18.5±2.6	19.1±1.6	18.1±2.2	18.9±0.8
		DI	21	16	21	18	19.0±2.5
		DF (%)	21	15	21	18	18.8±2.9
	APO	IL	21.4±3.1	20.8±2.0	19.5±3.3	19.2±1.8	20.2±1.0
		DI	24	33	30	31	29.5±3.9
		DF (%)	24	33	30	31	29.5±3.9*
	8-OASQ	IL	23.7±6.0	20.9±4.1	22.2±4.9	27.5±8.2	23.6±2.8*
		DI	71	71	68	111	80.3±20.6**
		DF (%)	58	67	58	73	64.0±7.4**
24[d]	Vehicle	IL	18.1±1.7	17.9±1.5	18.0±1.5	18.4±1.4	18.1±0.2
		DI	11	5	3	10	7.3±3.9
		DF (%)	11	5	3	10	7.3±3.9
	APO	IL	18.2±1.8	18.5±1.5	17.0±1.7	17.0±1.4	17.7±0.8
		DI	8	12	14	4	9.5±4.4
		DF (%)	8	11	14	4	9.3±4.3
	8-OASQ	IL	18.3±1.6	17.2±1.5	18.1±1.6	17.3±1.5	17.7±0.6
		DI	10	9	5	6	7.5±2.4
		DF (%)	10	9	5	6	7.5±2.4

[a] image length (IL); [b] damage index (DI); [c] damage frequency (DF).

[d] For positive control, at 3 h and 24 h sampling times, mice treated with 40 mg/kg MMS were killed and the peripheral blood and the brain tissue analyzed via Comet assay. The results for brain tissue were: IL: 27.1±4.2**: DI: 93.5±37.5**; DF: 41.0±5.7** (mean ±SD for two mice in 3 h sampling time) and IL:20.9±0.7**; DI: 33.5±3.5**; DF: 18.0±0* (mean ±SD for two mice in 24 h sampling time). Significant difference: *$p<0.05$; **$p<0.01$ (Dunnett test).

Table 2

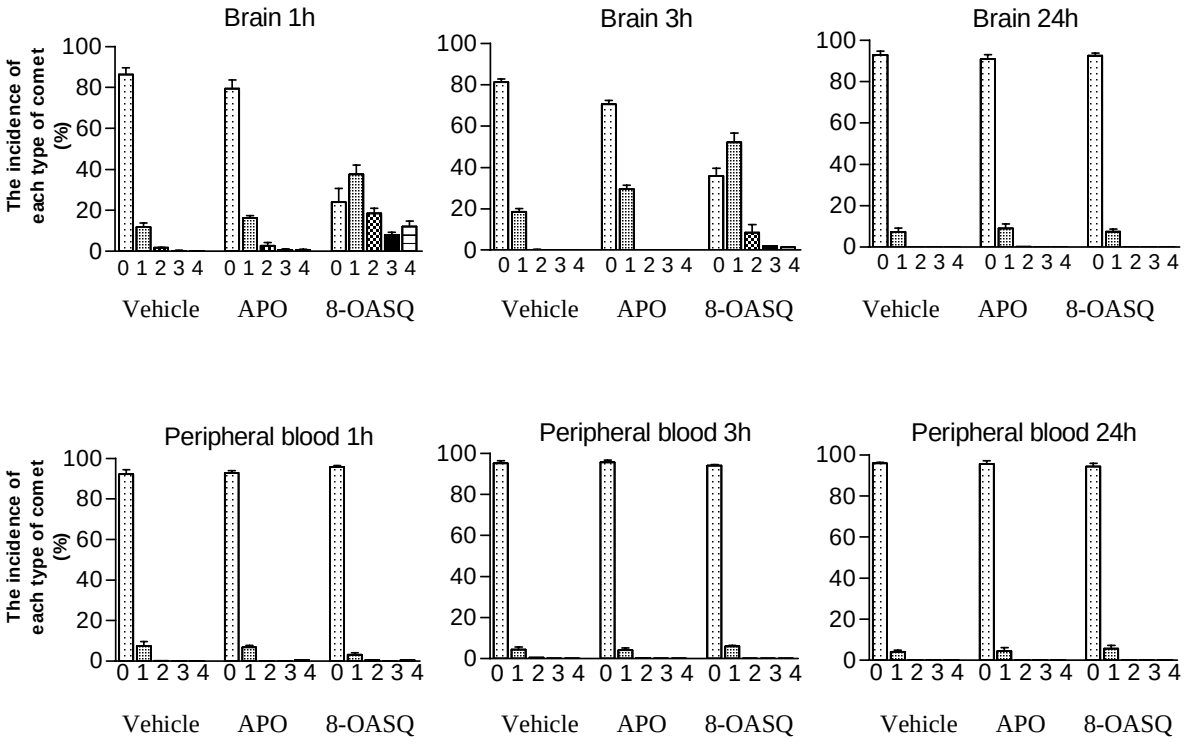
Comet assay in peripheral blood of mice treated with an i.p. injection of vehicle, apomorphine (APO; 40mg/kg), or 8-oxo-apomorphine-semiquinone (8-OASQ; 40 mg/kg), and sacrificed 1, 3 or 24 h after injection

Sampling time (h)	Group	Individual results					Mean±SD by group
1	Vehicle	IL[a]	16.9±2.4	15.7±2.1	21.8±4.2	20.5±2.4	18.7±2.9
		DI[b]	4	4	13	11	8.0±4.7
		DF[c](%)	4	4	12	11	7.8±4.4
	APO	IL	19.7±3.2	21.6±4.1	21.0±3.8	19.8±2.4	20.5±0.9
		DI	9	11	11	4	8.8±3.3
		DF (%)	9	8	8	4	7.3±2.2
	8-OASQ	IL	20.7±4.2	20.2±3.6	20.3±4.7	20.7±2.5	20.5±0.3
		DI	8	7	6	4	6.3±1.7
		DF (%)	5	6	3	3	4.3±1.5
3[d]	Vehicle	IL	18.9±1.8	18.6±1.5	17.3±1.8	17.9±1.3	18.2±0.7
		DI	9	6	4	3	5.5±2.7
		DF (%)	8	6	3	3	5.0±2.5
	APO	IL	18.6±1.4	20.1±2.0	17.2±1.8	18.3±1.3	18.6±1.2
		DI	3	9	3	4	4.8±2.9
		DF (%)	3	8	3	4	4.5±2.4
	8-OASQ	IL	19.0±1.6	17.5±1.0	18.8±1.6	17.7±1.5	18.3±0.74
		DI	6	6	7	5	6.0±0.8
		DF (%)	6	6	7	5	6.0±0.8
24[d]	Vehicle	IL	18.0±1.5	17.6±1.3	17.4±1.2	17.5±1.4	17.6±0.3
		DI	4	4	3	6	4.3±1.3
		DF (%)	4	4	3	6	4.3±1.3
	APO	IL	18.2±1.4	17.9±1.3	17.3±1.1	17.2±1.0	17.7±0.5
		DI	9	4	3	2	4.5±3.1
		DF (%)	9	4	3	2	4.5±3.1
	8-OASQ	IL	17.6±1.2	17.3±1.2	18.5±1.4	17.7±1.1	17.8±0.5
		DI	9	5	7	2	5.8±3.0
		DF (%)	9	5	7	2	5.8±3.0

[a] image length (IL); [b] damage index (DI); [c] damage frequency (DF).

[d] For positive control, at 3 h and 24 h sampling times mice treated with 40 mg/kg MMS were killed and the peripheral blood and the brain tissue analyzed via Comet assay. The results for peripheral blood were: IL:26.6±1.3**;DI: 100.0±7.1**; DF: 87.0±5.7** (mean ±SD for two mice in 3 h sampling time) and IL:24.3±2.4**; DI: 93.5±9.2**; DF: 43.0±9.9** (mean ±SD for two mice in 24 h sampling time). Significant difference: *$p<0.05$; **$p<0.01$ (Dunnett test).

Figure


Brain 1h
Brain 3h
Brain 24h
Peripheral blood 1h
Peripheral blood 3h
Peripheral blood 24h
The incidence of each type of comet (%)
0 20 40 60 80 100
0 1 2 3 4
Vehicle
APO
8-OASQ

VI. DISCUSSÃO GERAL

O processo neurodegenerativo da DP envolve uma cascata de eventos, incluindo danos oxidativos, disfunção mitocondrial (particularmente no complexo I), excitotoxicidade, desequilíbrio do cálcio, processos inflamatórios e apoptose. Estes eventos interagem e amplificam um ciclo vicioso de toxicidade, levando à disfunção neuronal e à morte celular (revisado em Dunnett e Björklund, 1999).

Uma das estratégias que busca diminuir o avanço da DP é a proteção dos neurônios dopaminérgicos, através da redução do estresse oxidativo. Para esta finalidade, são considerados promissores os fármacos que além de aliviar os sintomas da doença apresentem atividades que possam retardar a degeneração dos neurônios nigroestriatal dopaminérgicos, tais como atividades antioxidante, preventiva da geração de ERO, quelante de metais (especialmente ferro), e/ou indutora de enzimas antioxidantes (Chiueh et al., 2000).

A apomorfina (APO), apesar de utilizada há bastante tempo na DP, apresenta alguns efeitos especialmente relacionados à sua autoxidação que ainda não foram totalmente esclarecidos. A recente aprovação pelo FDA de APO no tratamento da disfunção erétil, aumentou a importância da determinação de possíveis efeitos genotóxicos. Os produtos de autoxidação de APO, que são gerados dentro de poucas horas após sua administração (Deleu et al., 2002), podem estar implicados em efeitos neurotóxicos, já relatados *in vitro* (Sit, 2000; El-Bachá et al., 2001). Outros estudos sugerem que APO possui atividades antioxidante e neuroprotetora (Chen et al., 2001; Battaglia et al., 2002b).

Neste trabalho, as atividades genotóxica, antimutagênica, citotóxica, pró/antioxidante da apomorfina (APO) foram avaliadas utilizando os testes *Salmonella*/microssoma, Mutoxiteste, e testes de sensibilidade em leveduras selvagem e deficientes em defesas antioxidantes (Capítulo I). Visando esclarecer a contribuição de efeitos dos produtos formados durante a autoxidação da APO nos sistemas biológicos, paralelamente, um produto da autoxidação da APO, 8-oxo-apomorfina-semiquinona (8-OASQ; Anexo I), foi também avaliado nesses experimentos (Capítulo I). Foram estudados os efeitos de APO e 8-OASQ na memória (Capítulo II) e no comportamento estereotipado (Capítulo III). A avaliação da atividade genotóxica foi complementada através do ensaio Cometa *in vivo*, para detectar a indução de possíveis danos ao DNA do tecido cerebral (Capítulo IV).

Como mostrado no Capítulo I, 8-OASQ induziu mutações por erro no quadro de leitura ("frameshift"), em linhagens de *S. typhimurium* TA98 e TA97 sendo que APO também apresentou esta atividade só que os índices de mutagenicidade foram aproximadamente duas vezes menores (Tabela 2). A oxidação de APO em produtos mais mutagênicos foi primeiro sugerido por Suter e Matter-Jaeger (1984), que também observaram indução de mutação "frameshift" de APO em *S. typhimurium* e atribuíram esta atividade aos seus produtos de autoxidação. A presença de S9 mix, aboliu o efeito mutagênico de ambos APO e 8-OASQ (Tabela 2). Duas hipóteses foram apresentadas para o efeito do S9 mix: o S9 mix poderia retardar a autoxidação de APO a 8-OASQ e outros derivados de degradação, devido à presença de enzimas e cofatores que ajudariam a manter a estrutura catecólica da APO; e/ou 8-OASQ e APO, poderiam ligar-se a proteínas do S9 mix, ficando indisponíveis para interagir com DNA. Prévios estudos mostraram que, principalmente os produtos oxidados de APO, ligam-se a

proteínas de forma inespecífica (El-Bachá et al., 1999), o que reforça a segunda hipótese.

Um dos assuntos que geram maior controvérsia em relação à APO é sua atividade como pró ou antioxidante. Normalmente as substâncias que se autoxidam apresentam uma destas atividades, dependendo das circunstâncias ou do ambiente em que se encontram. Por exemplo o pH, a presença de outras substâncias pró ou antioxidantes no qual o experimento é realizado, a dose utilizada, ou o estado redox do sistema biológico, são fatores que interferem nos resultados da atividade da substância como pró ou antioxidante.

Para tentar elucidar esses efeitos de APO em sistemas biológicos, APO e o produto oxidado 8-OASQ foram avaliados quanto a possíveis efeitos mutagênicos e antimutagênicos em linhagens de *S. typhimurium* TA102 e nas linhagens derivadas de WP2, IC188 (*uvrA*/ pKM101) e IC203 (*uvrA oxyR*/ pKM101), que são sensíveis a mutágenos oxidantes e a ERO (Mortelmans e Zeiger, 2000; Martínez et al., 2000). Uma resposta mutagênica mais alta para IC203 do que para a IC188 pode ser interpretada como indicador de mutagênese oxidativa, visto que ambas apresentam o mesmo "background" genético, com a exceção da deficiência de OxyR da IC203. OxyR é um fator de transcrição, que normalmente responde ao estresse oxidativo pela indução de enzimas antioxidantes (Ruiz-Laguna et al., 2000). A deficiência desse fator de transcrição aumenta a sensibilidade desta linhagem para avaliação de mutágenos oxidantes e geradores de ERO. A TA102 não é tão específica para avaliação de mutágenos oxidantes, e está também envolvida na avaliação de agentes "crosslink" e deletogênicos (Levin et al., 1982b; Picada et al., 1999). Outra diferença importante entre TA102 e as derivadas WP2 de *E. coli* é a capacidade de reparo por excisão-ressíntese

que está intacta na TA102 mas nas WP2 *uvrA* é defectiva (Mortelmans e Riccio, 2000; Mortelmans e Zeiger, 2000).

Os resultados apresentados na Tabela 5 do Capítulo I mostram que APO possui atividade antimutagênica para essas linhagens de bactéria, com percentuais de inibição próximos de 100%, na ausência de ativação metabólica. As mutações foram induzidas pelo peróxido de hidrogênio e terc-butil hidroperóxido, que são oxidantes geradores de ERO, sugerindo que a inibição da mutagenicidade pela APO possa estar associada a mecanismos antioxidantes. A presença de S9 mix prejudicou o efeito antimutagênico da APO (Capítulo I, Tabela 6), sugerindo que APO pode ligar-se a proteínas do S9 mix. Adicionalmente, APO protegeu as células de levedura do estresse oxidativo induzido por estes mesmos hidroperóxidos (Capítulo I, Figuras 2 e 3), um efeito que também pode ser relacionado com suas propriedades antioxidantes. É possível que APO tenha exercido uma atividade seqüestradora dos radicais hidroxil, gerado pelo H_2O_2 via reação de Fenton e peroxil, a partir da degradação do t-BOOH. Também é importante ressaltar que esse efeito foi observado inclusive em linhagens deficientes em defesas antioxidantes, o que aumenta a relevância da proteção pela APO, uma vez que normalmente as células neuronais de pacientes com DP apresentam deficiências nos mesmos mecanismos de defesa antioxidante das linhagens de levedura utilizadas, incluindo a diminuição dos níveis de glutationa reduzida (Sian et al., 1994; Youdim et al., 1999; Kunikowska e Jenner, 2001). A atividade antimutagênica sugere que APO poderia contribuir para a proteção do DNA contra os efeitos mutagênicos induzidos por ERO, que se encontram aumentados na DP (Dunnett e Björklund, 1999; Gerlach et al., 2000; Chiueh et al., 2000). O conjunto desses resultados corroboram outros modelos de estudo, que demonstram atividade antioxidante da APO. Em modelos de Parkinson, um

efeito neuroprotetor de APO parece estar relacionado com suas atividades "scavenger" (Fornai et al., 2001; Grunblatt et al., 2001a). Além disso, APO foi capaz de quelar ferro, diminuir a oxidação de ácidos graxos poliinsaturados e inibir a monoamina oxidase B (MAO-B), diminuindo assim degradação da dopamina e a geração de peróxido de hidrogênio (revisado em Youdim et al., 1999).

A forma oxidada 8-OASQ apresentou efeitos quase opostos aos observados pela APO. 8-OASQ induziu mutações por danos oxidativos nas bactérias sensíveis ao estresse oxidativo, especificamente as derivadas WP2 de *E. coli*, na ausência de S9 mix (Capítulo I, Tabela 5). Em levedura, 8-OASQ sensibilizou todas as linhagens, de forma dose dependente, principalmente as deficientes em catalase, superóxido dismutase e no fator de transcrição Yap1p (Capítulo I, Figura 1), sugerindo que 8-OASQ apresenta propriedades pró-oxidantes. APO induziu efeitos citotóxicos somente nas doses mais altas, nas quais foi sugerida a contribuição de formas oxidadas na produção dos efeitos deletérios (Capítulo I, Figura 1). A grande sensibilidade ao 8-OASQ das linhagens derivadas de EG103 isogênicas deficientes em catalase e superóxido dismutase citosólicas, indicam um importante papel dessas enzimas na inativação dos efeitos citotóxicos de 8-OASQ. A enzima superóxido dismutase está envolvida na dismutação do radical superóxido a peróxido de hidrogênio enquanto a catalase subseqüentemente converte o peróxido de hidrogênio a água e oxigênio molecular (ver Figura 5, pg.14). Tanto $O_2^{\bullet-}$ quanto H_2O_2 podem ser formados a partir de produtos de autoxidação de catecóis (Cheng et al., 1979; Kalyanaraman, 1990, Dunnett e Björklund, 1999). Portanto, o pronunciado efeito citotóxico de 8-OASQ nas linhagens simples mutantes Δ*sod1* e Δ*ctt1*, e ainda mais acentuado nas duplo mutantes Δ*ctt1*Δ*sod1* e Δ*sod1*Δ*sod2*, sugere o envolvimento das espécies $O_2^{\bullet-}$ e H_2O_2 e talvez de outras formas reativas no

processo deletério observado. A linhagem Δ*gsh1*, que é incapaz de sintetizar glutationa, foi utilizada para demonstrar o possível papel desse tripeptídeo no processo de proteção celular contra danos induzidos por APO e o produto oxidado 8-OASQ. A glutationa é o mais abundante tiol de baixo peso molecular presente nas células e está envolvida na manutenção do estado-redox celular, atuando também como co-fator para enzimas e como antioxidante (Meister, 1995). 8-OASQ foi mais deletério para a linhagem Δ*gsh1* do que APO (Figura 1), corroborando outros estudos que sugerem o envolvimento de glutationa e de outros tióis na prevenção tanto da autoxidação da APO quanto de danos celulares (Suter e Matter-Jaeger, 1984; El-Bachá et al., 2001). Outra linhagem que se mostrou bastante sensível, principalmente ao tratamento com 8-OASQ, foi a mutante Δ*yap1*. O fator de transcrição Yap1p parece regular uma série de genes em resposta ao estresse oxidativo, incluindo a maioria dos genes envolvidos na síntese de glutationa (Dormer et al., 2002). As mutantes Δ*yap1* são muito sensíveis a agentes oxidantes (Kuge e Jones, 1994; Stephen et al., 1995; Delaunay et al., 2000). O forte efeito na ausência do fator de transcrição Yap1p, indica a participação de sistemas de antioxidação celular na detoxificação de 8-OASQ, sugerindo novamente a formação de ERO a partir dos produtos de autoxidação da APO. Outros dois efeitos de 8-OASQ em levedura foram observados: primeiro, 8-OASQ, em doses não-citotóxicas, diminuiu a viabilidade das linhagens de levedura estressadas com hidroperóxidos, devido provavelmente à atividade pró-oxidante, e segundo, 8-OASQ propiciou um aumento da capacidade antioxidante das células de levedura ao posterior tratamento com os hidroperóxidos, devido possivelmente à indução de sistemas adaptativos (Capítulo I, Figuras 4 e 5). Este último efeito indica que os produtos de autoxidação de APO, em baixas concentrações, podem prevenir danos oxidativos, pela indução de fatores de

transcrição e/ou de enzimas que modulam o estado redox celular. A esse respeito, 8-OASQ apresentou um forte potencial antioxidante *in vitro* pelo ensaio TRAP ("total radical-trapping antioxidant potential") e foi capaz de aumentar os níveis de catalase em cérebro de camundongo (D.G. Flores e colaboradores, dados não publicados). Esses resultados sugerem uma possível contribuição de produtos oxidados de APO, favorecendo a neuroproteção.

Porém, prévios estudos mostram que APO e outros catecóis podem induzir neurotoxicidade mediada pelos derivados de autoxidação, quinonas e semiquinonas, e formação de ERO (Suter e Matter-Jaeger, 1984; Smythies, 1997; El-Bachá et al., 1999; 2001). O sistema nervoso central é geralmente considerado sensível aos danos oxidativos devido à sua elevada taxa metabólica e aos níveis altos de lipídeos insaturados (Liu et al., 2001). Além disto, as atividades de enzimas antioxidantes no cérebro são baixas comparadas a de outros órgãos. No cérebro, a elevação de ERO pode causar danos oxidativos em vários componentes celulares. Modificações químicas nas bases dos ácidos nucleicos, causam quebras de cadeia simples e duplas do DNA e formação de sítios AP (Liu et al., 2001).

Para avaliar os efeitos genotóxicos de APO e de 8-OASQ no cérebro, foi realizado o ensaio Cometa, que tem a vantagem de não necessitar de células em proliferação (Tice et al., 2000). APO induziu em pequeno mas significativo aumento na freqüência de danos ao DNA do tecido cerebral em relação ao grupo veículo (Capítulo IV, Tabela 1), a maioria danos de classe 1, que são os de menor magnitude (Capítulo IV, Figura 1). A dose foi de 40 mg/Kg i.p., aproximadamente dez vezes maior do que a dose diária utilizada para o tratamento de doentes de Parkinson (Deleu et al., 2002). O aumento na freqüência de danos foi observado após 3 h da administração, mas não após

1 h, indicando que é necessário um certo tempo para que o dano possa ocorrer, o que sugeriu um efeito devido a produtos de degradação. Sabe-se que no cérebro a concentração máxima de APO é atingida dentro de 10 a 20 minutos após sua administração sistêmica, e o seu desaparecimento ocorre rapidamente, dentro de 2 horas (Battisti et al., 2000c). El-Bachá e colaboradores (2000) demonstraram que APO não forma conjugados glucuronídeos no cérebro e sugeriram que o desaparecimento de APO no cérebro poderia ser devido à autoxidação. Por outro lado, 8-OASQ induziu danos ao DNA do tecido cerebral com pico máximo de 1 h após tratamento agudo na dose de 40 mg/Kg (Capítulo IV, Tabela 1). Provavelmente, os danos ao DNA induzidos por 8-OASQ foram devido aos seus mecanismos pró-oxidantes, que estão associados às reações redox geradoras de radicais quinonas e semiquinonas. Estes radicais promovem o aumento da formação do radical hidroxila, que é um potente indutor de quebras simples ao DNA (Brozmanová et al., 2001). Outro efeito de 8-OASQ que poderia estar relacionado aos danos observados ao DNA do tecido cerebral, é a possibilidade de sua intercalação com DNA, e/ou devido a uma reparação incompleta do DNA, gerando quebras. Surpreendentemente, os sistemas endógenos de detoxificação do animal não preveniram os danos ao DNA cerebral. *In vitro*, a presença de S9 mix, utilizado para reproduzir a metabolização de compostos pelo fígado, havia abolido o efeito mutagênico de 8-OASQ, tanto o "frameshift" quanto o oxidativo (Capítulo I).

Nenhum dano significativo permaneceu 24 h após o tratamento com APO ou 8-OASQ (Capítulo IV, Tabela 1), sugerindo a atuação de mecanismos de reparo do DNA. O principal mecanismo de reparo no cérebro parece ser o BER (reparo por excisão de base), que geralmente está associado à reparação de danos oxidativos (Liu et al., 2001).

Em amostras de sangue periférico dos mesmos animais, não foram observados danos significativos ao DNA, inclusive nos grupos sacrificados 1 h e 3 h após o tratamento com APO ou 8-OASQ (Capítulo IV, Tabela 2). A falta de efeito genotóxico em sangue sugere que houve ligação de APO e 8-OASQ com proteínas plasmáticas, causando inativação. O mesmo foi proposto para a falta de efeito de APO e 8-OASQ nos testes *in vitro*, em tratamentos na presença de ativação metabólica, neste caso, devido à ligação com proteínas do S9 mix (Capítulo I).

APO rapidamente entra no cérebro, onde fica acumulada por breve período logo após sua administração, atuando especialmente em receptores dopaminérgicos (Deleu et al., 2002). O sistema dopaminérgico desempenha importante papel sobre a memória, além de estar envolvido com funções motoras (Jang et al., 2002). Estudos em animais sugerem que prejuízos de memória e aprendizagem estão presentes na DP (Brown et al., 1997; Da Cunha et al., 2001); pacientes com DP mostram deficiências cognitivas, inclusive prejuízos de memória (Dubois e Pillon, 1997; Goldman et al., 1998), embora o sintoma clínico principal da DP sejam as perturbações motoras. Para estudar os efeitos cognitivos da APO e do produto de autoxidação 8-OASQ, animais tratados sistemicamente foram avaliados, na tarefa de esquiva inibitória (EI) e campo aberto (Capítulo II). Os efeitos comportamentais de APO corroboraram com estudos anteriores (Doyle e Regan, 1993; Doyle et al., 1996). APO provavelmente atuou sobre os receptores dopaminérgicos centrais, resultando em prejuízo na formação da memória de longa duração, na tarefa de EI, e na habituação não-associativa e não aversiva, sem afetar a memória de curta duração, locomoção, motivação, ansiedade ou nocicepção. Resultados diferentes foram observados para a forma oxidada da APO. 8-OASQ, prejudicou a retenção (memória) na tarefa de EI de curta e de longa duração, sem afetar a habituação, e os demais parâmetros de comportamento avaliados (Capítulo II),

sugerindo que 8-OASQ pode atuar sobre outros receptores. O conjunto desses resultados sugerem que os derivados de autoxidação de APO e possivelmente derivados de dopamina e L-DOPA, usados clinicamente no tratamento da DP, podem atuar sobre mecanismos envolvidos nos processos de aprendizagem e de memória e induzir deficiências cognitivas.

A investigação das ações comportamentais dos derivados de APO e outros agonistas dopaminérgicos pode auxiliar o entendimento dos mecanismos envolvidos nos efeitos de APO em pacientes com DP bem como os processos celulares e moleculares envolvidos em doenças psiquiátricas, como esquizofrenia, e na degeneração de neurônios dopaminérgicos associados com DP (Jang e Lee, 2001; Jang et al., 2002; Bonuccelli et al., 2002; Vivo et al., 2002). APO é utilizada como um modelo de indução de comportamento estereotipado, que está típicamente relacionado a um aumento da atividade agonista dopaminérgica, induzida por agonistas que atuam em receptores da dopamina ou por fármacos que indiretamente afetam a transmissão dopaminérgica (Moore e Grace, 2002; Ellenbroek e Cools, 2002; Estrella et al., 2002). No Capítulo III os resultados mostram que APO realmente induz de forma dose-dependente o comportamento estereotipado, provavelmente estimulando os receptores dopaminérgicos. Em contraste, a forma oxidada 8-OASQ não induziu este comportamento, sugerindo que a autoxidação da APO causa perda na habilidade de estimular diretamente esses receptores. É possível que as atividades neurobiológicas e/ou ações neurotóxicas de derivados de oxidação dos agonistas dopaminérgicos sejam mediados por mecanismos independentes da estimulação dos receptores dopaminérgicos. Muitos estudos têm sugerido que a neurotoxicidade da dopamina é mediada pela atividade de seus derivados quinona, não sobre os receptores dopaminérgicos, mas sobre os receptores glutamatérgicos NMDA, que podem levar à morte celular (Smythies, 1997; Jang e Lee, 2001; Vivo et al., 2002).

VII. CONCLUSÕES

1. Conclusão geral

APO e 8-OASQ revelaram efeitos distintos em quase todos os paradigmas utilizados neste estudo. O produto da autoxidação 8-OASQ apresentou efeitos genotóxicos mais pronunciados que APO, corroborando com diversos estudos que atribuem os efeitos deletérios de APO, e de outros agonistas dopaminérgicos, aos sub-produtos de sua autoxidação. Atividades antimutagênicas e antioxidantes de APO foram observadas em organismos procariotos (*Salmonella typhimurium* e *Escherichia coli*) e eucarioto (*Saccharomyces cerevisiae*), respectivamente. 8-OASQ apresentou efeitos mutagênicos por danos oxidativos em *E. coli* e propriedades pró-oxidantes em *S. cervisiae*. Uma resposta adaptativa contra agentes oxidantes em *S. cerevisiae* foi induzida pelo pré-tratamento com 8-OASQ, que aumentou a capacidade antioxidante das células de levedura. Efeitos genotóxicos em cérebro de camundongo foram claramente mais pronunciados pelo tratamento *in vivo* da forma oxidada da APO. 8-OASQ causou deficiências cognitivas em ratos diferentes daquelas produzidos pela APO, possivelmente através de mecanismos neurobiológicos distintos aos da APO e não foi capaz de provocar comportamento estereotipado em camundongo, sugerindo que a autoxidação da APO causa a perda de sua habilidade de exercer este efeito típico associado aos agonistas dopaminérgicos.

2. Conclusões Específicas

➢ APO e 8-OASQ são mutágenos por erro no quadro de leitura ("frameshift"), frente a linhagens de *S. typhimurium* TA98 e TA97, na ausência de ativação metabólica.

➢ 8-OASQ é um mutágeno indutor de danos oxidativos, frente a linhagens IC188 e IC203 de *E. coli*, na ausência de ativação metabólica.

➢ APO é antimutagênica devido a mecanismos antioxidantes, inibindo a atividade genotóxica de H_2O_2 e t-BOOH, induzida nas linhagens TA102, IC188 e IC203, na ausência de ativação metabólica.

➢ 8-OASQ é mais citotóxico que APO para linhagens de *S. cerevisiae*, especialmente deficientes no fator de transcrição Yap1p, glutationa e superóxido dismutase e catalase citosólicas.

➢ APO apresenta efeito protetor antioxidante para linhagens de *S. cerevisiae* selvagens e deficientes em sistemas antioxidantes.

➢ 8-OASQ apresenta efeito pró-oxidante e induz sistemas de adaptação ao estresse oxidativo, em linhagens de *S. cerevisiae* selvagens e deficientes em sistemas antioxidantes.

➢ APO prejudica a habituação a um novo ambiente e a memória de longa duração, sem afetar a de curta duração, na tarefa de esquiva inibitória, em ratos.

➢ 8-OASQ prejudica a retenção da memória de curta e de longa duração e não prejudica a habituação a um novo ambiente, em ratos.

➢ 8-OASQ não é capaz de induzir comportamento estereotipado em camundongos, que é tipicamente associado com a atividade agonista dopaminérgica, sugerindo que 8-OASQ não estimula receptores dopaminérgicos.

➢ 8-OASQ apresenta atividade genotóxica mais pronunciada que APO, para células do tecido cerebral de camundongo, avaliada pelo Ensaio Cometa.

➢ APO e 8-OASQ não apresentam atividade genotóxica para as células do sangue periférico de camundongo, avaliada pelo Ensaio Cometa.

VIII. PERSPECTIVAS

APO recentemente foi aprovada para uso no tratamento da disfunção erétil, o que ampliou muito a prescrição clínica deste fármaco, antes utilizado mais especificamente no tratamento da DP, em estágios avançados da doença. Poucos estudos foram realizados a respeito dos possíveis danos genotóxicos de APO e de seus produtos de autoxidação. APO exerce atividade dopaminérgica central, apresentando uma meia-vida muito curta no organismo, sendo que a autoxidação é uma das principais rotas metabólicas de seu desaparecimento, além de conjugação com sulfato e glucuronídeo, que parece ter pouca importância no cérebro (El-Bachá et al., 2000). Este trabalho mostra que um produto de autoxidação da APO apresenta efeitos deletérios mais pronunciados que a própria APO, induzindo inclusive danos ao DNA no cérebro de camundongos. Portanto, seria recomendável uma avaliação criteriosa do uso freqüente deste fármaco, devido aos possíveis efeitos genotóxicos, causados principalmente pelos seus produtos de degradação.

Adicionais estudos genotóxicos poderiam incluir o teste de micronúcleo e aberrações cromossômicas em roedores, utilizando protocolos de tratamento crônico, pela administração de APO e 8-OASQ, nas doses equivalentes as utilizadas nos tratamentos de doentes de Parkinson e também de disfunção erétil.

Os efeitos genotóxicos em cérebro de camundongo, induzidos por APO e 8-OASQ, representam apenas o início de um estudo que poderá ser ampliado pela avaliação desses efeitos em regiões alvo no cérebro, como o estriado e a substância negra.

Para complementar o estudo na levedura *Saccharomyces cerevisiae* seria importante analisar os efeitos de APO e 8-OASQ, nas linhagens mutantes deficientes em genes envolvidos no sistema antioxidante, utilizando as células na fase de crescimento estacionária, na qual há o envolvimento da mitocôndria na modulação da resposta ao estresse oxidativo e outros sistemas de detoxificação estão ativados, como a enzima superóxido dismutase mitocondrial (Mn-SOD) (Longo et al., 1999; Maris et al., 2000; 2001).

Com o propósito de esclarecer os efeitos de APO e 8-OASQ em diferentes regiões do cérebro, suas atividades neurobiológicas poderiam ser adicionalmente estudadas em um modelo animal de Parkinson induzido por ferro. Neste modelo, vários paradígmas poderiam ser avaliados: (1) as deficiências cognitivas (2) os efeitos genotóxicos em diversas regiões do cérebro, pelo ensaio Cometa e (3) o potencial antioxidante destas mesmas regiões, através de ensaios bioquímicos. Para uma melhor aproximação com o tratamento em humanos, os animais poderiam ser tratados continuamente com APO e 8-OASQ, via subcutânea, e nas doses equivalentes as utilizadas em pacientes com DP.

Outra proposta seria a avaliação de efeitos modulatórios sobre receptores NMDA glutamatérgicos por APO e 8-OASQ, pois alguns estudos sugerem que derivados quinonas e semiquinonas de agonistas dopaminérgicos poderiam atuar sobre esses receptores (Jang e Lee, 2001; Vivo et al., 2002). A excitotoxicidade, associada a estimulação de receptores NMDA, vem sendo apontado como um dos mecanismos que levam a morte de neurônios dopaminérgicos. O dano devido ao aumento dos níveis de glutamato, modifica a permeabilidade das células ao cálcio via ativação NMDA, intensificando o influxo de cálcio, que ativa a óxido nítrico sintetase (NOS),

aumentando a formação de óxido nítrico, que através de reação com radical superóxido gera peroxinitrito. Toda essa cascata de eventos aumenta o estresse oxidativo celular (Dunnett e Björklund, 1999). Seria muito importante analisar um possível envolvimento da APO e de seu produto de autoxidação neste processo.

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ANEXOS

ANEXO I

Dose finding in the Ames *Salmonella* assay

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Dose finding in the Ames *Salmonella* assay

Nikita N. Khromov-Borisov*,[1], Jaqueline N. Picada, João A.P. Henriques

Departamento de Biofísica e Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, RS, Brazil

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Abstract

Threshold dose/concentration values, such as the lowest effective dose, minimum effective dose or the lowest effective concentration (LED, MED or LEC, respectively) are in use as an alternative to the mutagen potency measures based on the 'rate' measurements (e.g., the slope of the initial part of the dose–response curve). In this respect, several statistical procedures for the corresponding so-called 'dose finding' were proposed during the last decades. However, most of them disregard the discrete nature of responses such as the plate colony count in the Ames *Salmonella* assay. When the plate counts agree with the Poisson assumption, two procedures considered here seem to be appropriate for the dose finding. One is based on the stepwise collapsing of the homogeneous control and dose counts; another consists of constructing the confidence limits for the mutation induction factor (MIF). When the dose and control counts are non-overlapping, the simple 'visual' non-parametric estimation of LED is possible. Applicability and validity of the methods is demonstrated with the two data sets on the mutagenicity of the β-carboline alkaloid, harmine, and one of the oxidation products of apomorphine.

Keywords: Ames *Salmonella*/microsome assay; Mutagen potency; Dose finding; LED — the lowest effective dose; Chi-square; Data collapsing; Statistical estimation; MIF — mutation induction factor; Confidence intervals; Alkaloids; Harmine; Oxidized apomorphine

1. Introduction

The problems of measurability and comparability are among the most provocative in science. In genetic toxicology, they manifest themselves by the absence of a universal measure of genetic potency and consequent uncertainty in the attempts to establish a predictive relationship between the various effects observed in different test systems (see e.g., [1–6]).

It seems reasonable to use the threshold dose/concentration values, such as the lowest effective dose, the minimum effective dose or the lowest effective concentration (LED, MED or LEC, respectively), instead of potency values based on the 'rate' measurements. These latter are commonly determined as the slopes of the initial (linear) part of the corresponding dose–response curves. For instance, "it may be possible for two compounds to have similar potency values but dramatically different LEDs (i.e., to be biologically active at different doses)" ([4], p. 280) or, in other words, "two dose–response curves may have the same initial slope, but may still be located at very different dose values" ([7], p. 159). Another reason is that "the biological effects observed in the different assays present largely heterogeneous metric and functional characteristics" ([7], p. 159). For example, for some effects, such as cytotoxicity, the potency measure analogous to the slope of the linear portion of the dose–response curves is absent. With

* Corresponding author. Tel.: +55-51-316-7608; fax: +55-51-316-7003/+55-51-319-1079.
E-mail addresses: nikita@dna.cbiot.ufrgs.br, pegas@dna.cbiot.ufrgs.br (N.N. Khromov-Borisov).
[1] On leave from Biological Institute of Saint-Petersburg University, Saint Petersburg, Russia.

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LEC, because it is not expressed in terms of biological effect, "... all potency measurements are put on a common metric scale (concentrations), which permit direct comparisons within and between different assay systems." ([7], p. 160). "Moreover, LEC is minimally distorted by the overlapping of mutagenicity with toxicity" ([7], pp. 159–160).

These circumstances determine the applicability of threshold doses such as the LEDs and the highest ineffective doses (HIDs) for the construction and analysis of computerized databases on genetic (or related) activity profiles (GAPs) [8]. "It should also be noted that recent analyses of *Salmonella* mutagenicity data revealed a significant negative relationship between LED (values from the EPA GAP database) and mutagenic potency (White and Rasmussen, unpublished results)" ([4], p. 280).

One of the problems in this respect is the choice of appropriate method(s) for the statistical estimation of LED. Benigni et al. who argued for the adequacy of LED as a potency measure in the investigation of relationships between in vitro mutagenicity assays, did not discuss this matter and presumably determined LEDs rather intuitively [7]. Fetterman et al. defined LED measured in the Ames *Salmonella* assay as "the lowest dose that has a mean colony count per plate significantly greater than the mean count per plate at the control dose", and assessed the significance "using a variance estimate based on the nonlinear dose–response model ... and a Bonferroni correction for multiple comparisons" ([5], p. 315), but they did not present any formulae and/or algorithms.

The testing approach employed to identify the lowest dose with an effect exceeding that of the control is known as the *dose-finding problem*. Several dose-finding procedures were proposed during the last decades and were reviewed and compared in the recent studies [9,10]. However, they disregard the discrete nature of the responses detected in some assays. Such procedures treat the responses as continuous, whereas in the main genetic toxicology test systems like plate colony counts in the Ames *Salmonella* assay, the responses are discrete. Meanwhile, discreteness bears additional information about the distribution of the counts, which can be checked with special statistical tests.

Here, we present three complementary statistical procedures based on our previous proposals and considerations concerning the Good Statistics Practice (GSP) [11] and Statistical Pattern Analysis (SPAN) [12]. They are illustrated with the two data sets on the mutagenicity of one of the oxidative products of apomorphine, 8-oxo-apomorphine-semiquinone (8-OASQ), and β-carboline alkaloid, harmine. Results on harmine were published previously [13] as a part of our systematic study of the genotoxic effects of alkaloids, which are sometimes very interesting and unusual [14–28]. Mutagenic activity of the isolated 8-OASQ is reported here for the first time.

2. Materials and methods

2.1. Chemicals and assay

Hydrochlorides of harmine [343-27-1] and apomorphine [314-19-*2*] were purchased from Sigma (St. Louis, MO, USA) and Merck KGaA (Damrstadt, Germany), respectively. 8-OASQ [24192-17-4] was isolated as a black water-insoluble precipitate after the incubation of the apomorphine hydrochloride (5 mg/ml) for 2 days and more in the phosphate buffer (0.02 M, pH 7.4) commonly used in the Ames test.

Mutagenicity testing was performed in accordance with the standardized protocols as recommended by Maron and Ames [29] and others [30–32]. Two *Salmonella typhimurium* strains, TA97 and TA98, were used. The preincubation procedure was applied where varying concentrations of a given alkaloid were preincubated with the cell suspension of a given tester strain for 20–30 min at 37°C either in the presence or absence of S9 mix.

2.2. Design of experiments and data presentation

One important peculiarity of our experimental design should be emphasized. As a rule, in each experiment, we used two negative controls: the medium control (MC) and the solvent control (SC). In MC, cells were suspended in buffer without any additives and preincubated before plating as described above. In SC, to the cells suspended in buffer, an appropriate amount of the solvent — DMSO (in the case of 8-OASQ) or water (in the case of harmine) — was added. No less than two plates were used in each control as well as at each dose level. As a rule, colony counts in both

controls were statistically homogeneous and collapsed (lumped) into one combined control count. It means that, after such collapsing, the number of plates in the combined control was twice of those used at different doses of the tested agent. From a statistical point of view, such a design of experiment leads to higher power of the test used and corresponds to known recommendations [30].

The semi-graphical format proposed earlier [11] was used for the data visualization as presented in Tables 2, 3 and 5. In such a format, all plate counts are shown and their ranking visualizes the dose–response pattern as well as the count variability at each dose level.

2.3. Statistical analysis

Statistical analysis was performed in accordance with GSP discussed earlier [11]. Like chemical structures [33], mathematical formulae are rare guests in biological texts. See, however, a good exception to this drawback [34]. Since to avoid confusion, we prefer to present here the main formulae used.

2.3.1. Notation

Here and thereafter, the plate counts observed in the controls and at each dose level will be called 'control counts' and 'dose counts', respectively. Let r denote the number of dose levels D_i applied (including two zero doses, D_M and D_S, in the controls, MC and SC); k_i is the number of plates examined at each ith dose level; n_{ij} is the plate count observed on the ijth plate, where $j = 1, 2, \ldots, k_i$; N_i is the sum of colony counts observed on the k_i plates at the ith dose level; K is the total number of plates used in a given experiment, and N is the total number of colonies observed on all K plates. R will denote the reduced number of groups of the control and dose counts obtained (instead of the initial number r) as a result of the collapsing procedure described below.

2.3.2. Visual estimation of LED

Often, the approach called earlier 'statistics in mind' [11] can be applied to estimate LED. It is based on the very simple criterion of overlapping or non-overlapping between different control and dose counts. However, it is applicable for the minimum number of plates shown in Table 1, which is an extract from tables for the critical values of the Wilcoxon–Mann–Whitney test statistic (see e.g., [11,35] among others). The two-sided tests should be used because we have no a priori consideration as to whether the tested agent be mutagenic or toxic. See also the argumentation in our paper on GSP [11]. For example, the three plates in the control and three at the nearest dose level are insufficient to achieve the nominal two-sided level α=0.05 (these numbers of the plates can provide the one-sided level $\alpha/2$=0.05 only). This is a statistical basis why we prefer to use three plates in both controls and at each dose level. In principle, they must be statistically homogeneous, and if so, the six plates in the combined (collapsed) control and three at the nearest dose level become sufficient to achieve the above level. Note that no adjustment for the multiple hypothesis testing is required in this case [10].

Actual (achievable) two-sided P-value can be easily obtained with the formula

$$P = 2\frac{\prod_{i=1}^{r} k_i!}{K!} \quad (1)$$

Table 1
The minimal (critical) numbers of plates in the control (k_j) and at the nearest dose level (k_i), which are required for the 'visual' estimation of LED

Significance level[a]											
0.1 (0.05)		0.05 (0.025)		0.02 (0.01)		0.01 (0.005)		0.002 (0.001)		0.001 (0.0005)	
k_i	k_j	k_i	k_j	k_i	k_j	k_i	k_j	k_i	k_j	k_i	k_j
1	19	1	39	1	99	1	199	1	999	1	1999
2	5	2	8	3	7	3	9	4	10	5	9
3	3	3	5	4	5	4	6	5	8	6	8
		4	4			5	5	6	7	7	7

[a] Two-sided significance level α. Values in parentheses represent one-sided significance level $\alpha/2$.

For example, in the above case of six plates in the combined control and three plates at the nearest dose level P=0.0119, if these counts are non-overlapping. The formula is valid for the non-overlapping dose counts only, otherwise other related procedures should be used [9–12].

2.3.3. Analysis of the raw data quality

The 'quality of the raw data' in the Ames *Salmonella* assay assumes homogeneity of plate counts within each dose count, that is, Poisson distribution for them [11]. An important aspect of the raw data quality is the homogeneity of counts in both controls used. As recommended [36], two kinds of hypotheses were tested:

- homogeneity of plate counts *within* each control and dose counts and
- homogeneity *between* the *mean* control and dose counts.

Respectively, two so-called 'Poisson variance tests' (or 'Poisson dispersion indices') were used:

$$X_i^2 = \frac{k_i}{N_i}\left(\sum_{j=1}^{k_i} n_{ij}^2\right) - N_i; \qquad \nu_i = k_i - 1 \tag{2}$$

and

$$X_I^2 = \sum_{i=1}^{r} X_i^2; \qquad \nu_I = \sum_{i=1}^{r} \nu_i = K - r \tag{3}$$

Both statistics are well known and used in genetic toxicology (see e.g. [37–39]). Calculation of the second one is realized in the GeneTox Manager software [40], but (as we found) is limited to a maximum of four plates per dose level. If the data are non-Poisson-distributed, the non-parametric inference should be applied (see [10,11]).

2.3.4. Collapsing and dose finding for the Poisson-distributed counts

When data agree well with Poisson assumptions, two complementary procedures for the dose finding can be used. The first approach is based on the stepwise (step-up) collapsing (lumping, combining, pooling etc.) of the most homogeneous subsets of data (control and/or dose counts). For this, the comparison between a pair of the mean values N_i/K_i and N_{i^*}/K_{i^*} observed at any two, ith and i^*th, dose levels is performed. Their difference $\delta_{i,i^*} = (N_i/k_i) - (N_{i^*}/k_{i^*}) = (k_{i^*}N_i - k_i N_{i^*})/k_i k_{i^*}$ is the natural measure of their similarity (distance) and the corresponding test statistic is the following χ^2:

$$X_{i,i^*}^2 = \frac{\delta_{i,i^*}^2}{\mathrm{Var}\delta_{i,i^*}} = \frac{K}{N}\frac{(k_{i^*}N_i - k_i N_{i^*})^2}{k_i k_{i^*}(k_i + k_{i^*})} \tag{4}$$

Asymptotically, each of the above statistics is χ^2-distributed with degrees of freedom $\nu_{i,i^*} = 1$.

Calculation of the X_{i,i^*}^2 values is performed for all possible pairs and presented as a triangle matrix of comparisons like those in Table 4. If one or more non-significant values are obtained, then the pair with the smallest value, which is the most homogeneous, is collapsed into single subset. The collapsing procedure is repeated until the step at which all X_{i,i^*}^2 values become significant. The minimal dose at which the response is statistically significant at the significance level α=0.05 should be regarded as the LED in agreement with the commonly accepted definition by Waters and co-authors [41] and others [5,9]. As will be shown below, in such an approach, there may be not a single LED value, but a group of mated doses due to the homogeneity of corresponding dose counts.

2.3.5. Constructing the simultaneous 95%-confidence intervals for MIF

The second procedure for the estimation of LED consists of constructing the *simultaneous* confidence intervals for the mutation induction factor (MIF) that is the ratio of the ith mean dose count, M_i=N_i/k_i, to the mean control count M_0=N_0/K_0:

$$\mathrm{MIF}_i = \frac{M_i}{M_0} = \frac{N_i k_0}{k_i N_0} \tag{5}$$

When the raw data agree with the Poisson assumption, MIF is a ratio of two Poisson variables. According to Goodman [42], the simultaneous lower and upper $(1-\alpha)\times 100\%$ confidence limits for such a ratio can be defined as

$$L_i^- = \mathrm{MIF}_i : \exp\left(z_{\alpha/2(R-1)}\sqrt{\frac{1}{N_0} + \frac{1}{N_i}}\right) \tag{6}$$

$$L_i^+ = \mathrm{MIF}_i \times \exp\left(z_{\alpha/2(R-1)}\sqrt{\frac{1}{N_0} + \frac{1}{N_i}}\right) \tag{7}$$

respectively, where $z_{\alpha/2(R-1)}$ is the critical value (percentile) of the standard normal distribution adjusted for the number of $(R-1)$ comparisons. As noted above, R is the (reduced) number of groups of the control and dose counts obtained after the appropriate collapsing of the initial r groups.

Much of modern statistical software allows one to find $z_{\alpha/2(R-1)}$ as well as the P-values associated with observed values of the above χ^2-statistics. However, we preferred to use two non-commercial software DSTATTAB [43] and ELV [44], which are referred to as being the most accurate [45].

3. Results

3.1. Visual estimation of LED

As the first example, the data on mutagenic activity of the one of oxidation products of apomorphine, 8-OASQ, in *S. typhimurium* strain TA97 without S9 mix are presented in Table 2 using a semi-graphical format proposed earlier [11].

Apomorphine, due to its structural and functional similarity with dopamine, has been clinically used as an emetic agent and in the treatment of Parkinson's disease. Previous works demonstrated that it acts either as an antioxidant or pro-oxidant. In *S. typhimurium*, its mutagenic activity was shown in frameshift-detecting strains TA97 and TA98 and was ascribed to its oxidation products [46]. It is known that the (aut)oxidation of apomorphine is pH-dependent and different products were detected in acidic and alkaline conditions [47]. Occasionally, we found that, under conditions of the standard Ames *Salmonella*/microsome assay (pH 7.4 and incubation for 48 h), apomorphine readily autoxidizes, yielding the derivative, 8-OASQ, which previously was attributed to the alkaline conditions only [47]. Thus, the mutagenicity study of this compound may be of high biological and medical importance.

In Table 2, one can see that all dose counts are non-overlapping except two controls. The last two are homogeneous according to the both the Wilcoxon–Mann–Whitney and the χ^2-tests. Consequently, they can be collapsed (combined). As a result, six counts in the combined control and three counts at the nearest dose are non-overlapping and significantly differ at $P=0.0119$. Thus, the first dose level, 10 μg per plate, is declared as the LED for 8-OASQ.

Other statistical procedures like those described in [9,10] or the procedure demonstrated below lead to the same conclusion. Only the power of the tests can be improved, resulting in the smaller P-values.

3.2. Raw data quality

The second example comprises the data on the mutagenic activity of harmine in *S. typhimurium* strain

Table 2
Visual estimation of LED: mutagenic activity of 8-OASQ in *Salmonella typhimurium* strain TA97 without S9 mix[a]

Dose (μg per plate)	Plate counts
60	558 612 621
40	419 463 487
20	321 357 363
10	***203 231 246***
0 (SC)	51 61 75
0 (MC)	70 80_2

[a] Subscript under the plate count (80_2) denotes the number of plates (two) with equal counts. MC and SC are the medium and solvent (zero doses) controls, respectively. Both control counts are collapsed into one due to their (obvious and statistically tested) homogeneity. Six control counts and three counts at the nearest dose, 10 μg per plate, are non-overlapping. According to the critical sample sizes in Table 1 they differ significantly at least at the two-sided level $\alpha=0.05$. According to formula 1 the actual P value is even less, $P=0.012$. The dose 10 μg per plate should be regarded as LED (data are emphasized in bold italics).

Table 3
Raw data quality analysis: mutagenic activity of harmine in *Salmonella typhimurium* strain TA98 in the presence of S9 mix[a]

Dose		Plate colony counts	Statistics				
i	D_i	n_{ij}	k_i	N_i	X_i^2	ν_I	P_i
7	250	32 39_2	3	110	0.89	2	0.64
6	200	28 30 34 35 40	5	167	2.61	4	0.63
5	150	28 30 31	3	89	0.16	2	0.92
4	100	22 23 33 37	4	115	5.73	3	0.13
3	50	24 26	2	50	0.08	1	0.78
2	0″	17 21	2	38	0.42	1	0.51
1	0′	16 21 22	3	59	1.05	2	0.59
Total			K=22	N=628	X_I^2=10.94	ν_I=15	P_I=0.76

[a] Notations correspond to those in Section 2. Subscript under the plate count (39_2) denotes the number of plates (two) with equal counts. Doses are expressed as μg per plate. Doses 0′ and 0″ correspond to medium and solvent controls (MC and SC), respectively. According to the *P*-values obtained the quality of the analyzed data does not contradict to Poisson assumptions and special powerful methods for Poisson-distributed data could be used for the further analysis and dose finding. Results are shown in Tables 4 and 5.

TA98 in the presence of S9 mix studied earlier [13] and presented in Table 3. The situation is more complicated than in the first example. Sample sizes (number of plates) at each dose level are unequal and the dose counts are overlapping, so visual estimation of LED is questionable. Statistical analysis of the internal homogeneity of replicate colony counts recorded at each dose level, using expression (2), have shown that all seven groups of replicate observations are homogeneous (see the values of X_i^2 and corresponding P_i in Table 3). Subsequent integrated test, using expression (3), also showed no extra-Poisson variation: X_I^2=10.94, ν_I=15 and P_I=0.76. This means that the quality of the data obtained does not contradict the Poisson assumptions and special powerful methods for Poisson-distributed data could be used for further analysis and LED estimation.

3.3. Dose finding

One method is the pairwise comparison between dose counts, using formula (4), and subsequent stepwise collapsing of the homogeneous pairs. Triangle matrix of the all pairwise comparisons between dose counts is presented in Table 4. Several pairs are homogeneous and they are emphasized in bold italics. To select what pairs should be collapsed, the stepwise collapsing described in Section 2 was used. According to it, the pair of both control counts with the smallest X_{i,i^*}^2 was collapsed first. The next was the pair of the dose counts observed at the doses 100 and 150 μg per plate. Then, these collapsed dose counts were collapsed with counts at the dose 50 μg per plate. The last collapsing was among the counts at two higher doses, 200 and 250 μg per plate. Resulting data are presented in Table 5. Only three groups of the dose counts produce significantly different groups, and the group 50+100+100 μg per plate is declared as the LED. Interestingly, the recent Chen's procedure [10]

Table 4
Pairwise comparisons and homogeneity between control and dose counts observed in the experiment with harmine[a]

Dose (μg per plate)	0 (MC)	0 (SC)	50	100	150	200	250
0 (MC)	***0***	***0.04***	***1.4***	5.8	6.2	14.5	17.8
0 (SC)		***0***	***1.5***	5.2	5.6	12.5	12.2
50			***0***	***0.8***	***1.1***	4.1	6.7
100				***0***	***0.06***	***2.0***	4.5
150					***0***	***1.1***	***3.0***
200						***0***	***0.8***
150							***0***

[a] The figures in the body of the table are the values of X_{i,i^*}^2. They should be compared with the well known critical value $\chi_{i,i^*}^2\{\nu_{i,j^*}=1;\ \alpha=0.05\}$=3.841. Statistically non-significant (P> 0.05) values are emphasized in bold italics. Corresponding pairs should be regarded as homogeneous and can be collapsed. The final result of the step-up collapsing see in Table 5.

Table 5
Final result of the step-up collapsing for the data on mutagenic activity of harmine, their quality analysis and dose finding[a]

Dose group		Plate colony counts	Statistics					
i	D_i (μg per plate)	n_{ij}	k_i	N_i	X_i^2	ν_i	P_i	$L^- <$MIF$< L^+$
3	200+250	28 30 32 34 35 39_2 40	8	277	4.04	7	0.78	1.5<1.78<2.06
2	***50+100+150***	***22 23 24 26 28 30 31 33 37***	***9***	***254***	***7.07***	***8***	***0.53***	***125<1.45<1.67***
1	0′+0″	16 17 21_2 22	5	97	1.51	4	0.83	0.85<1.00<1.17
Total			K=22	N=628	X_I^2=12.62	ν_I=19	P_I=0.86	

[a] Notations correspond to those in Section 2. Subscripts under the plate counts 21_2 and 39_2 denote the number of plates with equal counts. Doses 0′ and 0″ correspond to medium and solvent controls (MC and SC), respectively. For calculation the upper L^+ and lower L^- confidence limits for MIF of the values α=0.05, r=3 and, respectively, the value $z_{0.0125}$=2.241 were applied in the formulae (6) and (7). The last was obtained using software DSTATTAB [42] and ELV [43]. Dose group 50+100+150 μg per plate is regarded as LED (the data are emphasized in bold italics).

as well other preceding procedures [9] did not reveal the dose 50 μg per plate as the LED.

3.4. Constructing the simultaneous 95%-confidence intervals for MIF

Another method is the construction of simultaneous confidence intervals for MIF, using formulae (6) and (7), as described in Section 2. They are presented in the last column of Table 5. Because confidence limits for the MIF (observed at the dose group 50+100+150 μg per plate do not cover the value 1, this confirms the above conclusion about this group being the LED and enables one to visualize it.

4. Discussion

4.1. Visual estimation of LED

We believe that the procedure of 'visual' estimation of LED and supplementary Table 1 of the critical sample sizes are described and illustrated above rather clearly and do not require additional comments.

4.2. Raw data quality

Two diverse modes of statistical inference, model-based and non-model-based, are discussed in the literature concerning dose–response relationship and dose-finding problem [1–12,34,36–41,48]. The approach realized here can be regarded as being non-model-based; however, at least one statistical model is employed, the Poisson distribution for dose counts.

The phenomenon of the so-called 'overdispersion' or 'extra-Poisson variability' in colony plate counts is a serious problem in quantitative microbiology. Biometricians try to solve it inventing sophisticated statistical models for the phenomenon and corresponding tests (see e.g., recent review [48] and references therein and above). Introducing additional parameters in such models complicates the analysis and reduces its efficacy and reliability (see, however, discussion of related topics in [49]). Moreover, using different statistical models and methods may lead to different parameter estimates, and as a consequence, to different conclusions [11,48].

In our experiments, the variability of plate counts was in good agreement with the Poisson assumptions. In this respect, the impression gained is that "... if an experiment is carried out carefully to eliminate sources of variability as completely as possible, the data from the Ames *Salmonella*/microsome test follow Poisson distributions. A sophisticated and complicated statistical model is therefore not necessarily required to evaluate the test results" [38].

4.3. Dose finding for the Poisson-distributed plate counts

When data are discrete and agree with the Poisson assumptions the classical Pearson's χ^2 seems to be appropriate and reliable test statistic. It is well known

that when the counts are expected to be equal (as plate counts at each dose level) the χ^2-approximation is satisfactory for this statistic if $k_i \geq 3$ and $N_i \geq 10$ (see [50] and references therein). For the Ames test this is not a problem because almost always the individual counts at three plates are larger than 10.

The procedure proposed here is based on the proposition, which may be called the Principle of Collapsing [12,51] according to which statistically non-distinguished (homogeneous) data can be collapsed (that is lumped, or pooled, or combined etc.) and used as a whole and single data subset in the further analysis. It enables to combine (collapse) the statistically homogeneous plate counts that results in enhancement of the statistical power of the test. Analogous situation when appropriate collapsing leads to the improvement of the power of another statistical test has been recently discussed by Kaplan et al. [52]. Optimal collapsing procedure seems to be stepwise beginning from the most homogeneous pair of the dose counts and stopping under the step when the difference between all pairs becomes significant. Recently we have described such a procedure and supporting COLLAPSE software and showed their validity and applicability for the SPAN in mutational spectra [12].

Interestingly, as was shown, above in such approach the LED may be presented not only by a single value, but also by a group of mated doses if the corresponding plate counts appear to be homogeneous. No other procedures discussed in literature do take into account the possibility of existence for such internally homogeneous grouped LED [9,10].

The above procedure is supported with the alternative (complementary) method for constructing the simultaneous confidence limits for MIF based on the work of Goodman [42] (see also [11]). Recent comparative studies showed that Goodman's method has good statistical properties and performs well in most practical situations when the number of dose levels is greater than 2 and each plate count is greater than 5, provided the number of dose levels is not too large [53,54]. It gives results compatible with the above χ^2-tests (Table 5). An important advantage of confidence intervals consists in that they visualize recognition of the significant MIF values and the estimation of LED. When a confidence interval for a given MIF does not include unit, the hypothesis on the absence of mutation induction (MIF=1) should be rejected (see e.g. [11] and references therein) and the minimum dose which produces significant effect should be regarded as LED.

4.4. Further trends

One can agree with the recent notion that current protocols in genetic toxicology are not designed to determine the LED [6], but it seems they can be easily adapted and improved for that purposes. When in a preliminary experiment LED is estimated as a group of doses it might guide the experimenter how to modify the dose scale, intervals and range in further experiments to get a clear cut-off point (if that exists) or more narrow estimation.

LED might be used also as a basis for the semi-quantitative (qualitative) classification of the compounds. For instance when the observed effect appears to be statistically significant it might be considered as

- weak when LED is higher then 10 μg/ml,
- moderate when LED lies in the range 1–10 μg/ml,
- strong when LED lies in the range 0.1–1.0 μg/ml, and
- potent when LED is lower then 0.1 μg/ml.

So 8-OASQ can be regarded as moderate and harmine as weak mutagens. This might be alternative to the potency classification based on the order of magnitude of the studied effects [11,55].

Thus, in accordance with the previous statements [4,7] it seems reasonable to use the threshold concentration values such as LED, the lowest effective doses, along with other potency measures. "An extensive analysis of various potency measures of SAL (mutation in *Salmonella*) showed that the predictivity for qualitative or quantitative carcinogenicity of a number of different measures of SAL potency was only minimally more effective than using the qualitative results" [6]. Therefore, threshold doses can be useful in predictive studies.

Note added in proof

It should be noted that the dose finding procedures based on the partition of the total chi-square firstly was proposed by Lancaster in 1949 [56] and repeatedly advised by Cocharan [57].

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ANEXO II

Lista de trabalhos publicados durante o período de 1999-2003*

Boeira J.M., Viana A.F., Picada J.N., Henriques J.A.P. (2002) Genotoxic and recombinogenic activities of the two β-carboline alkaloids harman and harmine in *Saccharomyces cerevisiae*, Mutat. Res. 500: 39-48.

Pungartnik C., Picada J.N., Brendel M.B., Henriques J.A.P. (2002) Further phenotypic characterization of pso mutants of *Saccharomyces cerevisiae* with respect to DNA repair and response to oxidative stress, Gen. Mol. Res. 179-89.

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Picada J.N., Khromov-Borisov N.N., Henriques J.A.P. (1999) Deletogenic activity of 1,2:7,8-diepoxyoctane in the *Salmonella typhimurium* tester strain TA102, Mutat. Res. 437: 165-173.

*Não foram incluídos na lista os artigos que fazem parte desta tese.