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Aline Steinmetz

Investigação dos mecanismos de citotoxicidade da cocaína utilizando células de glioblastoma em cultura

Porto Alegre
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Dissertação submetida ao Programa de Pós-Graduação em Patologia da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

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“Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.”

Charles Chaplin

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A todos que, de alguma forma, colaboraram para tornar possível este trabalho.

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Lista de Abreviaturas

7AAD: 7-Aminoactinomicina D

8-OHdG: 8-hidroxi-2' -desoxiguanosina

ATP: Trifosfato de Adenosina (do inglês, *Adenosine triphosphate*)

Bcl-2: B-cell lymphoma 2

BZE: Benzoilecgonina

CAT: Catalase

CEBRID: Centro Brasileiro de Informações sobre Drogas

D1: Receptor dopamina 1

DCF: 2',7'-diclorofluoresceina

DCFH-DA: 2',7'–diclorofluorescina diacetato

DCT: Túbulo contorcido distal (do inglês, *distal convoluted tubule*)

DNA: Ácido desoxirribonucleico (do inglês, *deoxyribonucleic acid)*

EC: Ecgonina

EME: Ester metilecgonina

FPG: Enzima de reparo de lesões específicas no DNA (do inglês, *formamidopyrimidine DNAglycosylase)*

GSH: Glutationa

HPTECs: Células do epitélio tubular (do inglês, *human primary tubular epithelial cells*).

IL: Interleucina

i-NOS: do inglês, *inducible nitric oxide synthase*

IUPAC: International Union of Pure and Applied Chemists

LDH: Lactado desidrogenase (do inglês, *lactate dehydrogenase*)

MTT: 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio

NADH: do inglês, *nicotinamide adenine dinucleotide*

NCOC: Norcocaina

NF-KB: do inglês *Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells*

NOX2: NAPDH Oxidase 2

PCT: Túbulo contorcido proximal (do inglês, *proximal convoluted tubule*)

PeQui: Perfil Químico

PE: do inglês, *phycoerythrin*

ROS: Espécies Reativas de Oxigênio (do inglês, *reactive oxygen species*)

SNC: Sistema Nervoso Central

SOD: Superóxido dismutase

SOX_2: do inglês, *Sex Determining Region Y-box 2*

TBARS: Substâncias Reativas ao Ácido Tiobarbitúrico (do inglês, *thiobarbituric acid reactive substances*)

UNODC: Escritório das Nações Unidas sobre Drogas e Crime (do inglês, *united nations office on drugs and crime*)

Estrutura da Dissertação

Esta dissertação foi estruturada da seguinte forma: introdução geral, justificativa do trabalho, objetivo geral e objetivos específicos, um artigo de revisão bibliográfica, o qual compõe o capítulo 1, e um artigo de dados, como capítulo 2. Na parte final da dissertação constam uma discussão geral, as conclusões e as perspectivas.

Na introdução são apresentados fatos históricos relacionados à cocaína, desde seu uso remoto na civilização andina até os dias atuais. Também são apresentadas características químicas da droga e sua composição, bem como dados epidemiológicos e aspectos relacionados à sua toxicocinética e toxicodinâmica.

O capitulo 1 traz o artigo de revisão bibliográfica a ser submetido à *Toxicology in vitro.* Neste trabalho, foi realizado um levantamento de dados presentes na literatura relacionados à toxicidade *in vitro* e *in vivo* da cocaína em diferentes sistemas e órgãos, como o cardiovascular e o sistema nervoso central.

No capítulo 2 é apresentado o artigo de dados do trabalho desenvolvido durante o mestrado a ser submetido à *Chemico-Biological Interactions*. Foi realizada avaliação dos mecanismos de toxicidade da cocaína em células imortalizadas de glioblastoma e em células de cultura primária de astrócitos. Para tal, após exposição das células à cocaína durante 24h, realizamos testes para verificar a viabilidade celular, a indução de formação de ROS e de danos ao DNA, a perda do potencial mitocondrial e a morte celular por apoptose e/ou necrose.

A discussão geral faz uma avaliação e comparação dos dados observados no artigo de revisão com aqueles obtidos após os testes de citotoxicidade realizados durante o desenvolvimento do mestrado. Finalmente, apresentamos uma conclusão sobre o trabalho desenvolvido e perspectivas para trabalhos futuros.

Resumo

A cocaína é utilizada como droga de abuso devido seus efeitos psicoestimulantes. Atualmente, é uma das drogas de abuso mais consumida no mundo, perdendo apenas para a maconha. Devido seu grande potencial aditivo, tornou-se um problema de saúde pública mundial. No presente trabalho, no capítulo 1 realizou-se uma revisão bibliográfica dos principais trabalhos realizados até o momento relacionados à toxicidade da cocaína em modelos in vivo e in vivo.

No capítulo 2, avaliou-se os mecanismos de toxicidade da cocaína (padrão e apreendida) em células de glioblastoma e em células de cultura primária de astrócitos. Para tal, realizamos testes de viabilidade celular utilizando os ensaios de Azul de Tripan e MTT após exposição das células a cloridrato de cocaína por 24 e 72 horas (2-15mM). Além disso, também foi avaliada a viabilidade celular após as células serem expostas à cocaína de apreensão por 24 e 72h utilizando ensaio de MTT com intuito de comparar os efeitos da droga pura com os efeitos da droga que é usualmente encontrada nas ruas. Além dos testes de viabilidade, também se verificou a ocorrência de formação de espécies reativas de oxigênio, por meio do ensaio de diclorofluoresceína (DCF) e, paralelamente, foi avaliada a atividade das enzimas superóxido dismutase (SOD) e catalase (CAT). A indução de danos ao DNA foi verificada por meio do ensaio cometa, versão alcalina e modificada. Para avaliar a perda do potencial de membrana mitocondrial, realizou-se o ensaio de Rodamina-123. Por fim, avaliou-se a indução de morte celular (apoptose e necrose) por meio da marcação celular com Anexina-V e 7AAD.

Neste trabalho ficou demonstrado que a cocaína, tanto a droga padrão quanto a droga de apreensão, é capaz de reduzir a viabilidade celular de forma dose e tempo dependente. Também foi observado que a cocaína leva a formação de espécies reativas de oxigênio, induz a morte celular por apoptose e por necrose, além de induzir danos a macromoléculas, como quebras na molécula de DNA e a peroxidação de lipídeos de membrana. Os dados relativos ao tipo de morte induzida, evidenciaram que a cocaína de apreensão induziu mais morte por necrose do que a cocaína padrão, fato que poderia estar relacionado às impurezas presentes neste tipo de amostra. Após exposição à cocaína por 24h, ocorreu a perda de potencial de membrana mitocondrial devido a sua hiperpolarização. Os resultados obtidos sugerem a relação entre a formação de espécies reativas de oxigênio e a toxicidade da cocaína descrita na literatura. A exposição das células a concentrações crescentes da droga induziu a formação de ROS, consequência que parece ser fator desencadeante para as ocorrências seguintes como indução de dano no DNA, hiperpolarização da membrana mitocondrial e, consequentemente, indução de morte celular.

Abstract

Cocaine is used as a drug of abuse because of its psychostimulant effects. It is currently one of the most commonly abused drugs in the world, behind only to marijuana. Due to its great additive potential, it has become a worldwide public health problem. In the present work, in chapter 1 we performed a bibliographical review of the main studies related to cocaine toxicity in vivo and in vivo models.

In Chapter 2, the mechanisms of cocaine toxicity (standard and seized) were evaluated in glioblastoma cells and astrocyte primary culture cells. For this, we performed cell viability tests using the Tripan Blue and MTT assays after exposing the cells to cocaine hydrochloride for 24 and 72 hours (2-15 mM). In addition, cell viability was also assessed after cells were exposed to seizure cocaine for 24 and 72 h using MTT assay in order to compare the effects of the pure drug with the effects of the drug that is usually found on the streets. In addition to the viability tests, the formation of reactive oxygen species was verified through the dichlorofluorescein assay (DCF) and, in parallel, the activity of the superoxide dismutase (SOD) and catalase (CAT) enzymes was evaluated. Induction of DNA damage was verified using the comet assay, alkaline and modified version. To evaluate the loss of mitochondrial membrane potential, the Rhodamine-123 assay was performed. Finally, the induction of cell death (apoptosis and necrosis) was assessed by cell-binding with Annexin-V and 7AAD.

In this work, it was demonstrated that cocaine, both the standard and the seized drug, is able to reduce cell viability in a dose-dependent and time-

dependent manner. It was also observed that cocaine leads to the formation of reactive oxygen species, induces cell death by apoptosis and necrosis, and induces damage to macromolecules, such as breaks in the DNA molecule and the peroxidation of membrane lipids. The data from the induced death type showed that seized cocaine induced more necrotic death than standard cocaine, a fact that could be related to the impurities present in this type of sample. After exposure to cocaine for 24 hours, loss of mitochondrial membrane potential occurred due to its hyperpolarization. The results obtained suggest the relationship between the formation of reactive oxygen species and cocaine toxicity described in the literature. The exposure of cells to increasing concentrations of the drug induced ROS formation, a consequence that seems to be a triggering factor for the following occurrences as induction of DNA damage, hyperpolarization of the mitochondrial membrane and, consequently, induction of cell death.

1 Introdução

1.1 Aspectos históricos

Erythroxylon coca é uma planta nativa da América do Sul, possuindo mais de 200 espécies diferentes, sendo a cocaína o principal alcaloide presente nela. O uso das folhas (figura 1) faz parte da cultura dos povos dos Andes, remontando a séculos passados e está envolvido com práticas de curandeirismo e com lendas relacionadas à fertilidade e a morte, sendo considerada sagrada para a civilização Inca (Ferreira et al., 2001; Biondisch et al., 2016).

Mesmo após a conquista espanhola, a coca continuou sendo utilizada pelos índios, especialmente devido a força e resistência que sua utilização gerava para que suportassem o trabalho árduo a que eram submetidos durante a colonização, posteriormente o hábito de mascar as folhas também se tornou comum entre os colonizadores (Chasin et al., 2008). Na Europa, o uso da folha de coca se popularizou em meados de 1860, quando folhas da planta eram utilizadas para fazer misturas com vinho. Embora amplamente consumido em alguns países, não houve relatos de toxicidade relacionados ao consumo, muito provavelmente devido à baixa concentração de cocaína na bebida (Karsch, 1996).

Figura 1: Folhas de *Erythroxylon coca.* Adaptado de Restrepo, 2007

Em 1884, o médico Sigmund Freud escreveu um artigo intitulado Uber coca, no qual descreveu os hábitos de uso da folha de coca por índios que habitavam a região dos Andes, e como essa prática estava relacionada a situações onde lhes era exigida maior força ou resistência nas práticas do dia a dia, até mesmo suportando por dias seguidos abstenção de alimentos.

Em experiências realizadas, em si mesmo e em outras pessoas, o médico relatou “o maravilhoso efeito estimulante da coca”, citando efeitos como euforia duradoura, aumento do autocontrole, maior vigor e capacidade para o trabalho e, por fim, ressaltando seus efeitos analgésicos, descobertos por Karl Koller (Freud, 1884).

Com a produção de cocaína semi-refinada em diversos países, a droga passou a estar presente em diferentes produtos da indústria farmacêutica sem nenhuma lei ou fiscalização para a produção ou consumo desses produtos, o que colaborou para que, algumas décadas após, houvesse relatos de intoxicações e mortes devido ao uso frequente desses produtos se tornassem cada vez mais corriqueiras em periódicos médicos (Ferreira, 2001).

Durante o século XIX, a cocaína foi amplamente comercializada, sendo encontrada nas ruas de forma fácil e barata. A indústria Parke Davis Company, industrializava a droga para uso terapêutico em 15 formas diferentes, dentre elas em cigarro e em uma formulação pronta para injeção. Mais adiante a cocaína foi incluída na formulação da Coca-Cola, criada por Jonh Styth Pemberton (Karch, 1999) e, algumas décadas depois, foi feita a substituição do xarope que continha o alcalóide por um extrato das folhas da planta sem cocaína (Chasin, 2008).

Em 1914 uma lei federal norte Americana – Harrison Narcotics Tax Act - que regulava e taxava a produção, importação e distribuição de determinadas drogas, passou a incluir a cocaína, devido suas já conhecidas propriedades aditivas. No Brasil, o decreto-lei Federal nº 4.292 de 6 de julho de 1921, estabeleceu penalidades para quem vendesse cocaína (Ferreira, 2001). A partir da década de 20 o uso da droga sofreu um declínio, muito em parte devido a popularização de anfetaminas entre os usuários (Goldstein, 2009).

Já na década de 70, o abuso de cocaína volta a crescer tendo grande influência da cultura, hábitos da época, como festivais de música e também incentivados por anúncios em revistas que vendiam utensílios para facilitar o consumo da droga, bem como encorajavam seu uso. Acredita-se que fatores econômicos também impulsionaram o aumento do consumo de crack, uma das formas de apresentação da cocaína, conhecida por ser absorvida rapidamente pelo organismo, atingindo os efeitos desejados em segundos (Karch, 1996).

1.1 Epidemiologia

A produção global de cocaína, em 2014, foi estimada em cerca de 943 toneladas, sendo a principal rota de tráfico vinda da sub-região dos andes para América do Norte e Europa. Entre 2009 e 2014, a Colômbia foi a responsável por 56% da cocaína apreendida na América do Sul, seguida por Equador e Brasil na segunda e terceira posição, respectivamente (World Drug Report, 2016 - UNODOC). Segundo o relatório, no ano de 2014 somava-se mais de 18 milhões de usuários de cocaína no mundo.

O Relatório Europeu sobre Drogas de 2017 aponta que a cocaína é o estimulante ilícito mais consumido na Europa, cerca de 3,5 milhões de adultos fizeram uso no último ano. Tal fato, movimenta um mercado de cerca de 5,7 milhões de euros, sendo responsável por 9% do total de apreensões, perdendo apenas para *cannabis* com 70%.

No Brasil, em 2005, a prevalência sobre o uso de Cocaína, Crack e Merla (uma forma de apresentação da cocaína feita com a pasta base e utilizada na forma fumada) era de 2,9%, 0,7% e 0,2 %, respectivamente (II Levantamento Domiciliar Sobre Uso de Drogas Psicotrópicas no Brasil – CEBRID). Nas 108 cidades com mais de 200 mil habitantes incluídas no levantamento, a prevalência de uso na vida de cocaína foi de 2,9% dentre os 7.939 entrevistados. Dentre os entrevistados, 0,5% entre 12 e 17 anos e 5,2% entre 25 e 34 anos já fizeram uso da droga na vida, com maior predomínio entre os homens. A região sudeste teve maior prevalência de uso na vida de cocaína com 3,7%, seguida pele região Sul em segundo lugar com 3,1%, Centro-Oeste com 2,3% e nas regiões Norte e Nordeste próximo a 1% em cada

uma delas. A região Norte foi a que apresentou o maior uso na vida de Merla, 1,0 %. Já o Crack teve o maior uso na vida no Sul (1,1%), seguido pelo Sudeste com 0,8 %.

1.2 Cocaína

A cocaína, cuja nomenclatura da IUPAC é Metil-(1S, 3S, 4R, 5R)-3-(benzoiloxi)-8-metil-8-azabiciclo[3.2.1]-octano-2-carboxilato, é um dos alcalóides encontrados nas folhas da *Erytroxylum coca* e *Erythroxylum novogranatensis* (Biondich, 2016). Sua estrutura química consiste de um grupo lipofílico, um grupo hidrofílico e um grupo alifático que se liga aos dois primeiros (Figura 2)

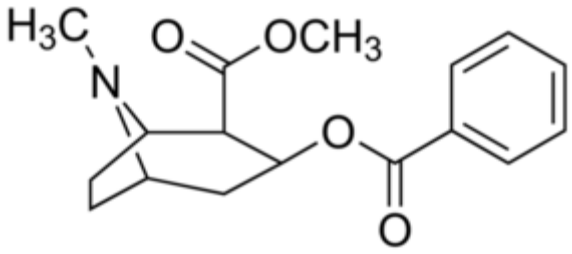

Figura 2: Fórmula estrutural da cocaína. Fonte: www.sigmaaldrich.com

Dentre as diferentes formas de apresentação de cocaína encontradas, as mais comuns são a pasta base, o cloridrato de cocaína e o crack (Fig 3). Durante a extração de cocaína das folhas de *Erythroxylon coca*, são empregados solventes orgânicos, como gasolina, bases inorgânicas ou ácidos. Como produto dessa extração, é obtida a pasta base de cocaína, que se

apresenta na forma de pequenos grumos ou pedras (Vargas, 2009; Ferigolo, 2007). Nessa forma, ainda estão presentes muitas impurezas utilizadas nesse processo. Em seguida, a pasta base de cocaína é dissolvida em ácido sulfúrico diluído e posteriormente adicionada uma solução oxidante de permanganato de potássio para eliminar as impurezas provenientes da primeira fase, o produto desse processo é chamado de cocaína base (Junior, 2012).

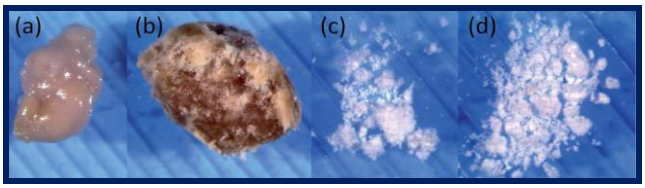

Figura 3: Formas de apresentação da cocaína: (a) Merla, (b) Crack, (c) Pasta base e (d) Cloridrato de cocaína. Fonte: Marcelo et. al, 2016.

O cloridrato de cocaína é produzido a partir da adição de ácido clorídrico à pasta base, obtendo-se um pó branco cristalino como resultado final (Figura 4). Sua utilização mais comum é a forma aspirada, mas também pode ser injetado a partir de uma solução aquosa. Já o crack, em forma de pedra, é obtido da adição de uma substância básica, mais comumente bicarbonato de sódio ou amônia, ao cloridrato de cocaína (Ferigolo, 2007). Por possuir baixo ponto de fusão, é a forma utilizada para fumar (Passagli, 2009; Ferigolo, 2007). Das formas menos comuns da droga, encontra-se ainda a Merla, uma pasta branca com alto teor de impurezas e também consumida na forma fumada.

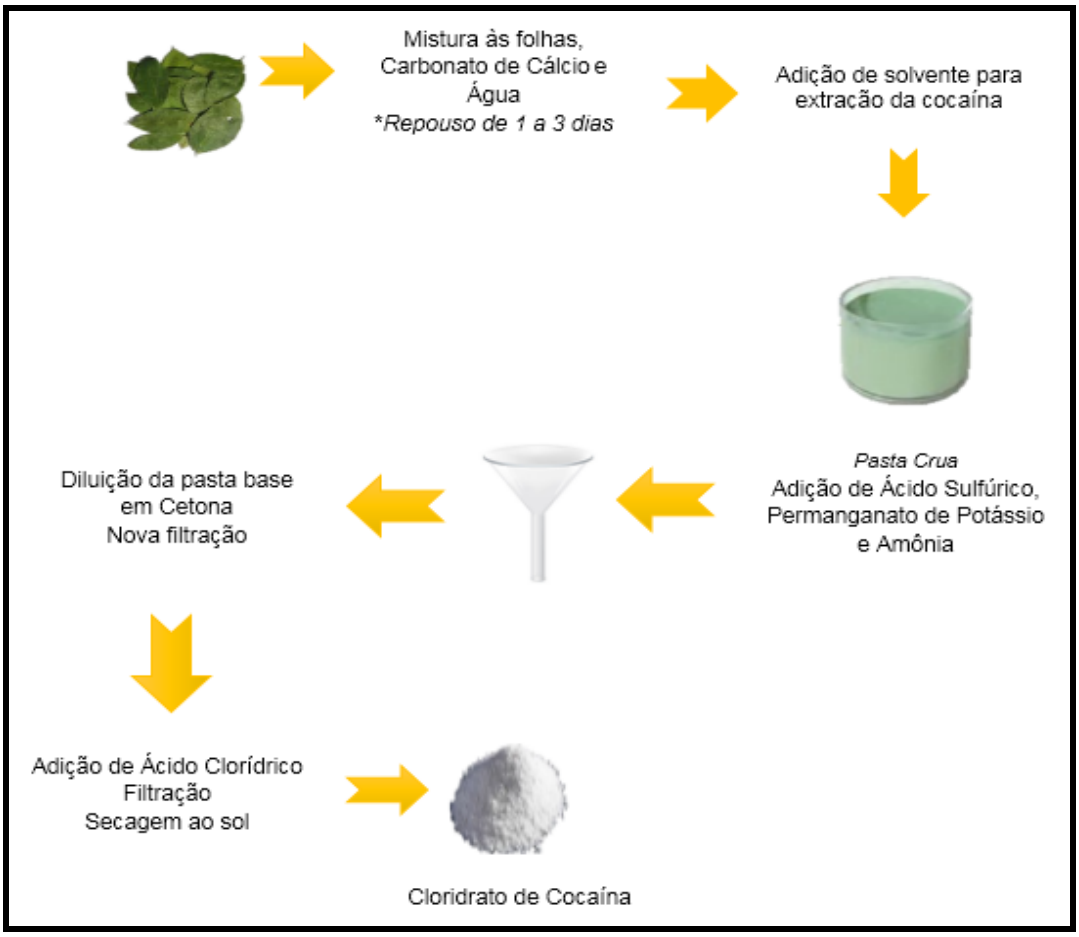

Figura 4: Esquema processo de produção das diferentes formas de cocaína

1.2.1 Contaminantes

Durante o processo de produção de cocaína, antes que ela chegue as ruas, são adicionados a sua composição adulterantes que, muitas vezes contribuem com os efeitos tóxicos decorrentes do abuso da droga. Tais substâncias podem ser utilizadas com intuito de aumentar o volume final da droga e, consequentemente, o lucro, como no caso da adição diluentes, como talco ou farinha. Ainda, há adição de outras substâncias com ação farmacológica semelhante que são utilizadas para potencializar o efeito da

droga ou sua toxicidade, dentre elas a cafeína, lidocaína, fenacetina e o levamisol são encontrados com frequência em drogas de apreensões (Lapachinske et al., 2014).

Atualmente, muitos casos de agranulocitose severa com necrose têm sido relatados em usuários de cocaína em diversos países. Tal fato associa-se a presença de levamisol utilizado na adulteração da cocaína. Esse anti-helmíntico, amplamente utilizado no passado para tratamento de artrite reumatoide, foi retirado do mercado norte americano e canadense devido ao grande número de casos de agranulocitose relatados como efeito adverso (Ittiachen et al., 2015; Desviqnes et al., 2017). Estima-se que quase 70% da cocaína que entra nos Estados Unidos está contaminada com levamisol, enquanto que no Reino Unido, o fármaco foi encontrado em mais de 50% das amostras de drogas analisadas (Lee et al., 2012).

O programa intitulado Projeto PeQui da Polícia Federal brasileira faz análise das drogas apreendidas a fim de identificar características que possam correlacionar as apreensões realizadas e a proveniência da droga, auxiliando assim no combate ao tráfico nacional e internacional. Uma análise de perfil químico de cocaína apreendida proveniente de 8 estados brasileiros entre os anos de 2009 e 2012 demonstrou que as drogas apresentaram um grau de pureza acima de 70%.

Embora os padrões de adulteração mudem de acordo com a região em que a droga foi apreendida, bem como sua forma de apresentação, os adulterantes mais encontrados nas drogas analisadas pela Polícia Federal foram cafeína, lidocaína e fenacetina (Zacca et al., 2014). Outro estudo utilizando 58 amostras apreendidas no estado do Rio Grande do Sul entre 2013

e 2015 encontrou como únicos contaminantes a cafeína, fenacetina e levamisol, sendo este último somente encontrado nas amostras de cloridrato de cocaína (Marcelo et al., 2016).

1.2.2 Toxicocinética

A cocaína pode ser utilizada por via oral, aspirada, injetada ou fumada, sendo que o tempo de absorção, o pico de concentração plasmática e a metabolização variam de acordo com a rota administrada. A via oral costuma ser a mais lenta e com efeitos menos intensos devido ao metabolismo de primeira passagem que a droga sofre e à baixa biodisponibilidade, enquanto que a fumada possui absorção mais rápida e os efeitos desejados sentidos em poucos minutos (Jones et al., 1997). Assim como a forma fumada, a cocaína utilizada de forma endovenosa também leva aos efeitos desejados de forma rápida, uma vez que a droga é injetada diretamente na corrente sanguínea.

Embora a via nasal seja a rota em que os efeitos sejam sentidos por um período mais longo, a absorção também sofre resistência devido a vasoconstrição causada pela própria droga (Ferigolo et al., 2012). Um estudo conduzido com voluntários que receberam cocaína comparou as vias nasal e oral, concluindo que a principal diferença é que a nasal induz concentrações sanguíneas mais altas de forma mais rápida que a via oral, embora 70% da cocaína administrada por essa última rota atinja a circulação (Fattinger et al., 2000). Outro estudo realizado com usuários de cocaína que receberam a droga por via nasal, fumada e endovenosa também avaliou a farmacocinética da cocaína. Com as vias endovenosa e fumada, efeitos como aumento da pressão

sistólica e diastólica foram observados imediatamente. O autor observou que benzoilecgonina, principal metabólito da cocaína, foi detectado no plasma num mesmo intervalo de tempo, entre 15 e 30min após administração, para as três rotas acompanhadas. A média de biodisponibilidade da cocaína fumada foi de 70% enquanto que da via intranasal foi superior a 90%, indicando que quase a totalidade da droga administrada foi absorvida para circulação sistêmica de forma inalterada (Cone, 1995).

Quase que a totalidade da cocaína absorvida sofre biotransformação no plasma ou no fígado, apenas cerca de 5% da droga é eliminada inalterada. A cocaína, após absorvida, é biotransformada em ester metilecgonina (EME) e benzoilecgonina (BE) (Figura 5). Uma série de estudos já demonstrou a ação das colinesterases plasmáticas, convertendo cocaína em EME. Os autores observaram que o fígado também possui atividade enzimática capaz de hidrolisar cocaína em ester metilecgonina, inclusive com velocidade maior do que as esterases plasmáticas, embora essas possuam maior afinidade com a cocaína (Inaba, 1978; Stewart, 1979).

BE é o principal metabólito encontrado na urina, produzido pela ação de esterases hepáticas, possui meia vida de 5 a 6 horas (Goldstein, 2009). Além desses metabólitos, outros também são gerados durante a biotransformação da cocaína, dentre eles a ecgonina (EC) e a norcocaína (NCOC). A norcocaína é um metabólito ativo, produzido a partir da N-demetilação da droga pela ação da isoenzima CYP3A4 (Valente, 2011) e possui atividade semelhante à cocaína, inibindo a recaptação da dopamina (Hawks, 1974).

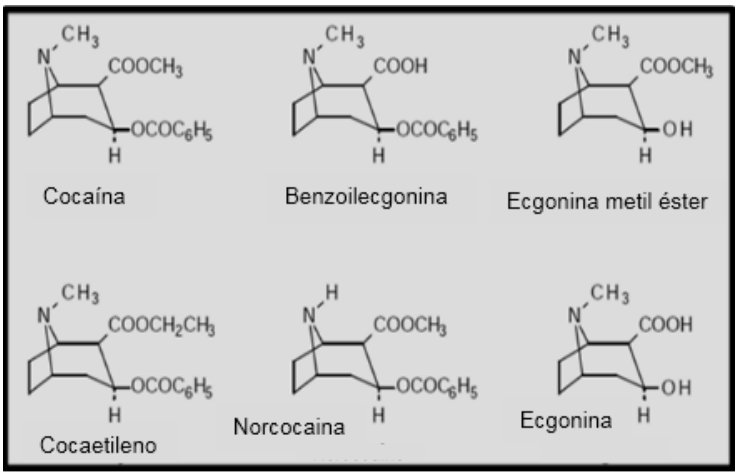

Figura 5: Principais metabólitos da cocaína. Adaptado de Harrison, 2007.

1.2.3 Toxicodinâmica

A cocaína possui ação psicoestimulante atuando na inibição da recaptação de catecolaminas, dentre elas serotonina, norepinefrina e dopamina nas fendas sinápticas. A dopamina e a via de sinalização do receptor D1 parecem estar ligadas ao sistema de recompensa relacionado ao desenvolvimento de adição (Hummel, 2002). Além disso, ocorre o bloqueio dos canais de sódio, o que resulta em seus conhecidos efeitos anestésicos locais. Efeitos como aumento da pressão arterial, taquicardia e euforia são comumente observados no uso agudo, enquanto que no uso crônico citam-se alucinações, anorexia, distúrbios cardiovasculares e respiratórios, rabdomiólise entre outros (CEBRID). Diversos estudos têm demonstrado que a cocaína pode causar toxicidade em diferentes sistemas do organismo, como o sistema nervoso central (SNC), o cardiovascular e o respiratório (Aquaro, 2011; Drent, 2012; Riezzo, 2012).

1.3 Metodologias utilizadas neste estudo

Para facilitar o entendimento dos resultados apresentados neste trabalho optou-se por trazer uma fundamentação teórica das metodologias utilizadas para obtenção dos resultados.

1.3.1 Ensaios de Viabilidade Celular

Ensaios de citotoxicidade são utilizados para determinar os efeitos que uma substância específica é capaz de produzir em determinado sistema ou órgão-alvo. As células expostas a um agente tóxico costumam responder de forma rápida à agressão sofrida, alterando funções vitais para sua sobrevivência como manutenção das membranas celulares e taxas de crescimento e metabolismo, podendo levar a ativação de vias de morte celular, como a apoptose (Eisenbrand et al., 2002).

Uma das características fundamentais para manutenção da homeostase celular é a integridade da membrana plasmática. Um dos ensaios mais utilizados para verificar a viabilidade celular é o método de azul de tripan, considerado uma técnica simples e rápida. Quando a célula perde sua integridade de membrana, o corante é capaz de penetrar no citoplasma e esse parâmetro é utilizado para diferenciar as células viáveis das não viáveis (Avelar-Freitas et al., 2014).

Outra técnica amplamente utilizada para análise de viabilidade celular é o ensaio de redução do MTT (brometo de 3-(4,5-dimetiltiazol-2-yl)-2,5-bifenil tetrazolio) levando a formação de formazan (Figura 6), cuja absorção é

proporcional a quantidade de células viáveis. Após exposição à substância em estudo, as células são incubadas com uma solução de MTT por cerca de 3 horas, seguindo então para leitura por espectrofotometria. Além da mudança na absorbância, nota-se visualmente a formação de uma coloração violácea nos poços da placa, cuja intensidade da cor é proporcional ao número de mitocôndrias viáveis (Riss et al., 2013).

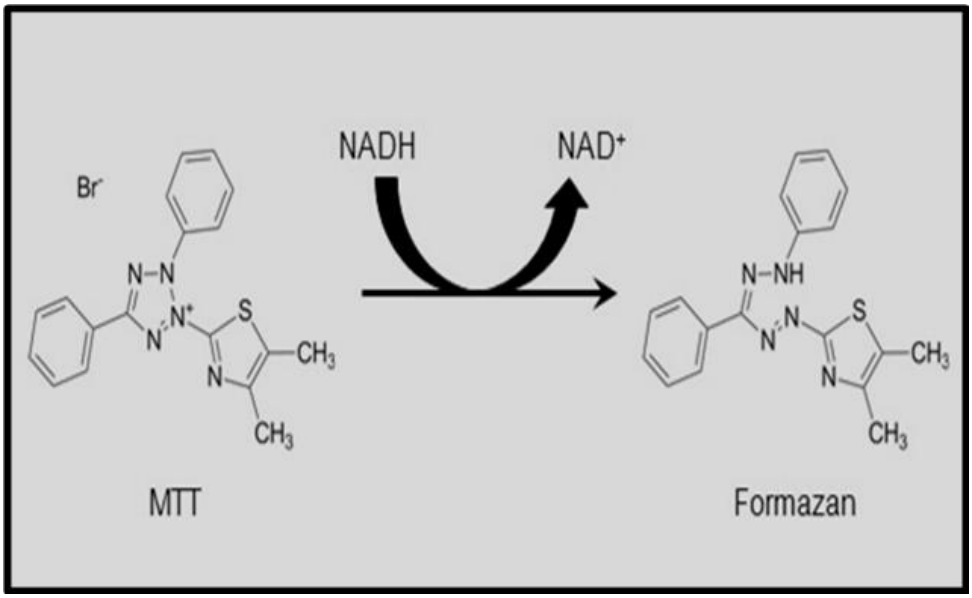

Fig 6: Reação de redução do MTT e formação de formazan. Adaptado de Riss, 2013.

1.3.2 Genotoxicidade – Ensaio Cometa

O ensaio cometa, desenvolvido nos anos 80, é um método simples, rápido e econômico utilizado para avaliar danos no DNA. Após o rompimento das membranas celulares, as lâminas pré-cobertas com agarose contendo um volume de suspensão celular são submetidas a eletroforese em condições alcalinas. Havendo quebras na molécula de DNA, as regiões danificadas

migram em direção ao ânodo durante a passagem de corrente elétrica, formando assim as caudas que serão posteriormente, ao microscópio, visualizadas e classificadas (5 classes, sendo zero, quando não há quebras no DNA e não se visualiza cauda e 4, quando praticamente todo DNA está em cauda) (Figura 7). Para possibilitar a visualização do DNA ao microscópio, comumente se utiliza brometo de etídio ou nitrato de prata como corantes. Além da versão alcalina, há uma versão modificada que utiliza enzimas capazes de aumentar a especificidade e sensibilidade do teste, como a Endonuclease III (Endo III), capaz de detectar pirimidinas oxidadas e a formamidopirimidina(fapi)-DNA glicosilase (FPG), que detecta purinas oxidadas (Collins, 2004). Esta versão modificada é utilizada para diferenciar se o dano observado é uma consequência direta da exposição ao agente tóxico ou proveniente de danos oxidativos ou sites purínicos/pirimidínicos (Smith, 2006).

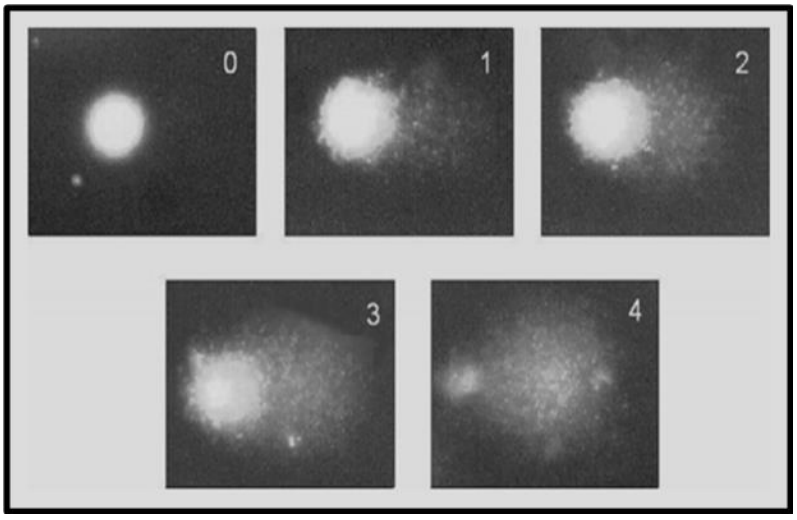

Fig 7: Classificação dos danos no DNA (0-4). Adaptado de Collins, 2004.

1.3.3 Formação e Detecção de Espécies Reativas de Oxigênio (ROS)

A geração de ROS tem sido associada a diversas doenças degenerativas como aterosclerose, Parkinson e inflamações crônicas, sendo que os efeitos do estresse oxidativo gerado, por vezes, acabam induzindo morte celular programada (Uttara et al., 2009). Sendo assim, um método para avaliar a ocorrência de ROS em modelos *in vitro* e *in vivo* é de extrema importância (Wang, 1999). O ensaio de DCFH-DA (diacetato de diclorofluoresceína) é considerado um teste fácil e rápido, sendo amplamente utilizado para detecção de espécies reativas de oxigênio. Basicamente, DCFH-DA, ao cruzar as membranas celulares, é hidrolisado enzimaticamente por meio de esterases intracelulares a DCFH não fluorescente (Figura 8). Na presença de ROS, DCFH é oxidado a 2',7'-diclorofluoresceina (DCF), que é fluorescente (LeBel, 1990).

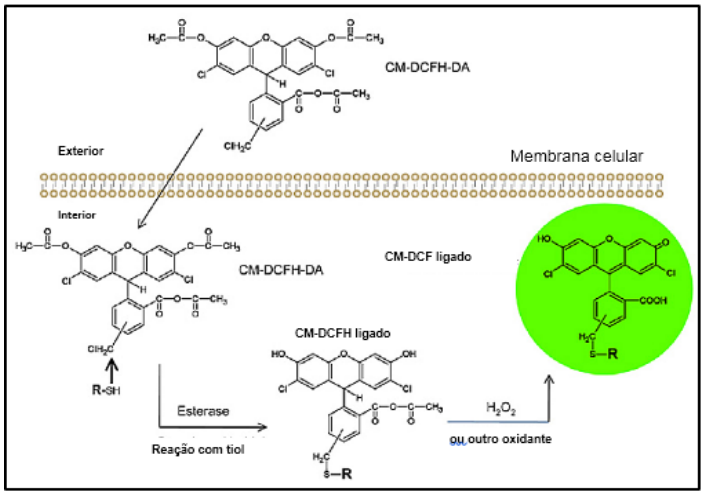

Fig 8: Esquema de DCF. Fonte: genomics.unl.edu

1.3.4 Indução de Apoptose

A avaliação de morte celular pode ser realizada utilizando-se marcadores de apoptose e necrose, como por exemplo Anexina V-PE e 7-Aminoactinomycin D (7AAD) por meio de citometria de fluxo (Figura 9). Durante a apoptose, ocorre externalização de fosfatidilserina de membrana no qual a proteína Anexina V é capaz de se ligar. Em estágios de apoptose tardia ou de necrose, devido a perda da permeabilidade da membrana celular, o marcador nuclear fluorescente Iodeto de Propídio (PI) é capaz de intercalar com ácidos nucleicos, exibindo assim fluorescência (Rieger, 2011). Já 7-AAD é um corante que se liga a regiões do DNA. Ele é utilizado para diferenciar células viáveis de células apoptóticas, baseado na permeabilidade da membrana celular. Dessa forma, a fluorescência será baixa nas células que ainda estão em apoptose inicial, e alta nas células em apoptose tardia ou mesmo nas mortas (Zembruski et al., 2012).

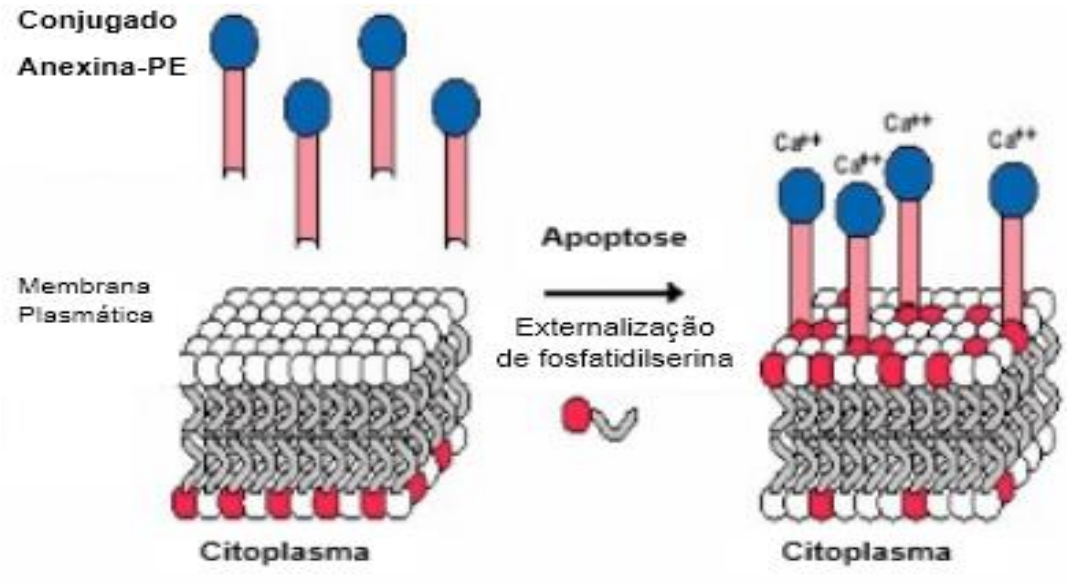

Fig 9: Marcação com Anexina-PE. Fonte: eukaryoticgeneexpresision.weebly.com

1.3.5 Potencial de Membrana Mitocondrial

Dentre os métodos empregados para avaliar o potencial de membrana mitocondrial, o corante fluorescente Rodamina-123 é amplamente utilizado por ser especifico para células vivas e não causar toxicidade celular (Angrimani, 2015). Este método se baseia no aumento de fluorescência induzido por sua acumulação eletroforética na mitocôndria (Baracca, 2003). As células são incubadas com Rodamina-123 por 30 min e posteriormente a fluorescência emitida é medida por citometria de fluxo.

1.3.6 Atividade Enzimática - Superóxido dismutase (SOD) e Catalase (CAT)

A presença de radicais livres em excesso pode levar ao estímulo de reações em cadeia, interagindo com lipídeos, proteínas e ácidos nucléicos, fato que leva ao estresse oxidativo e pode culminar com a morte celular (Rukmini, 2004). As enzimas superóxido dismutase (SOD) e catalase (CAT) fazem parte do sistema de defesas antioxidantes do organismo contra os danos que radicais livres podem causar (Figura 10). Estas enzimas são utilizadas como biomarcadores da presença de estresse oxidativo (Radovanovik et al., 2010). SOD atua convertendo radical superóxido em peróxido de hidrogênio e oxigênio. Já a catalase atua convertendo o peróxido de hidrogênio em água e oxigênio (Weydert, 2009).

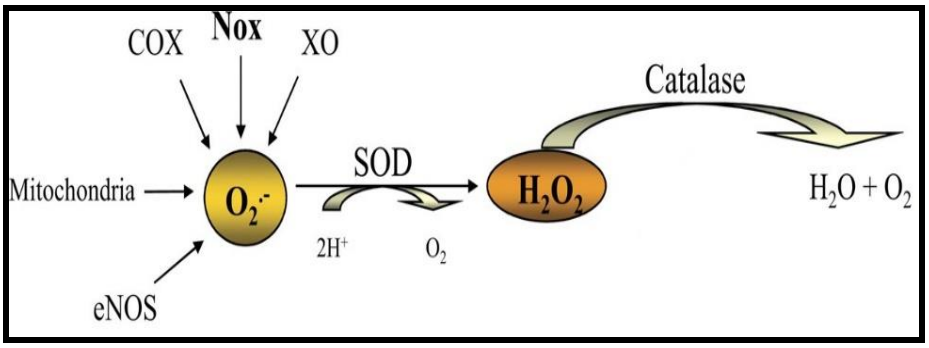

Fig 10. Atuação das enzimas antioxidantes superóxido dismutase e catalase. Adaptado de Frazziano et al., 2012.

3.6 Peroxidação Lipídica – Ensaio TBARS (espécies reativas ao ácido tiobarbitúrico)

A peroxidação lipídica está associada a patogenia de diversas doenças como diabetes, Parkinson e Alzeheimer. Ela ocorre na oxidação de lipídeos que contém ligações duplas entre carbonos (Figura 11), como os lipídeos que compõem as membranas celulares, por exemplo, de forma que o dano gerado a partir desse mecanismo representa um alto risco para a homeostase celular e sua sobrevivência (Devasagayam, 2003). Malondialdeído (MDA) é um dos principais produtos formados durante a oxidação de cadeias de ácidos graxos insaturados, sendo utilizado como indicador de ocorrência de peroxidação lipídica (Grotto, 2009). Resumidamente, o MDA reage com ácido tiobarbitúrico (TBA) formando substâncias reativas ao TBA (TBARS) de coloração rosa/avermelhada cuja absorbância pode ser lida por espectrofotometria (Siddique, 2002).

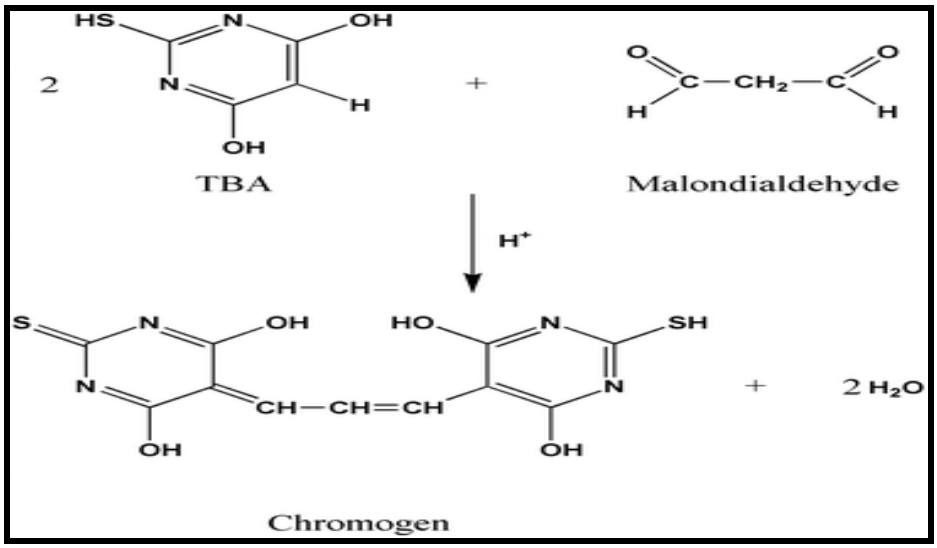

Fig 11. Reação de formação de espécies reativas ao ácido tiobarbitúrico.
Fonte: Antolovich, 2001.

2. Justificativa

A cocaína é a segunda droga de abuso mais utilizada no mundo todo, sendo responsável por um grande número de internações em hospitais decorrentes do seu abuso. Tal fato é considerado um problema de saúde pública e vem desafiando autoridades e entidades ligadas ao controle e prevenção de seu uso. Na literatura, diversos trabalhos vêm demonstrando os danos potenciais que a exposição à cocaína é capaz de causar tanto em modelos animais quanto *in vitro*. Em vista disso, o objetivo deste estudo foi propor um modelo *in vitro* para avaliar a toxicidade da cocaína que agregue os diferentes parâmetros de toxicidade já observados em estudos anteriores, porém de forma isolada. A perspectiva deste trabalho é que este modelo possa ser utilizado para avaliação da cocaína apreendida pelos órgãos responsáveis e possa auxiliar no entendimento dos efeitos relacionados à toxicidade observados nos usuários, em especial, pela presença dos diferentes contaminantes.

3. Objetivos

3.1 Objetivo Geral

Estabelecer um modelo *in vitro* para avaliação da citotoxicidade da cocaína em cultura celular de glioblastoma, através da investigação da formação de radicais livres, indução de lesões na molécula de DNA e definição do tipo de morte celular.

3.2 Objetivos Específicos

1- Determinar o perfil de citotoxicidade da cocaína utilizando os ensaios de MTT e Azul de Tripan.

2- Deteminar o tipo de morte celular utilizando os marcadores anexina V PE e 7-AAD por citometria de fluxo.

3- Verificar formação de quebras na molécula de DNA utilizando o ensaio cometa alcalino e a versão modificada do teste cometa para verificação da formação de lesões oxidativas no DNA.

4- Verificar a ocorrência de peroxidação lipídica por meio do ensaio TBARS.

5- Verificar a formação de espécies reativas de oxigênio induzida por cocaína utilizando o marcador fluorescente diacetato de diclofluoresceína e análise por citometria de fluxo.

6- Verificar atividade das enzimas Superóxido Dismutase (SOD) e Catalase (CAT);

7- Verificar a alteração da função mitocondrial induzida por cocaína utilizando o marcador fluorescente rodamina-123 e análise por citometria de fluxo.

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Toxicity of cocaine through *in vitro* and *in vivo* studies: an overview

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Abstract

Cocaine is a psychoactive alkaloid extracted from the leaves of *Erythroxylum coca*. Cocaine abuse is an important public health problem, being the second drug of abuse most utilized worldwide. The drug is a stimulant of the central nervous system and act by blocking the reuptake of catecholamines at the synaptic cleft. As acute effects, tachycardia, rise in arterial blood pressure, and arrhythmias are common, besides euphoria and addiction. Several studies have reported the toxic effects of cocaine in various organs and systems, as heart, kidney, lung and brain. The major cause of cocaine toxicity seems to be due the generation of reactive oxygen species (ROS), inducing oxidative stress. A great number of *in vitro* and *in vivo* studies reported that the exposure to different concentrations of cocaine can induce the formation of ROS. As a consequence, DNA damage, loss of mitochondrial membrane potential, and cellular death through apoptosis were observed. This review emphasizes the current knowledge on the cocaine toxicity using *in vitro* and *in vivo* models.

Keywords: cocaine, contaminants, toxicology

Abbreviations: ATP: Adenosine triphosphate, Bcl-2: B-cell lymphoma *2*, BE: benzoylecgonine, CNS: central nervous system, COMT: cathecol-o-methyltransferase, DAT: dopamine transporters, DEA: Drug Enforcement Administration, DNA: deoxyribonucleic acid, ED1: dopamine receptor, EME: ecgonine methyl ester, GSH: Glutathione, HPTEC's: human proximal tubule primary culture, LDH: lactate dehydrogenase, MAO: monoamine oxidase, NADH: Nicotinamide adenine, dinucleotide, NCOC: norcocaine, NF-KB: nuclear factor *kappa B*, PC12: phaeochromocytoma cells, PTP: permeability transition pore, ROS: reactive oxygen species, SOD: superoxide dismutase enzyme, SOX2: Sex Determining Region Y-box 2, TBARS: Thiobarbituric acid reactive substances.

1 Introduction

Cocaine is an alkaloid obtained from the *Erythroxylum coca* leaves, a native plant of South America. Its use appears from the sixth century, where it was used in religious rituals. The latest World Drug Report from the United Nations Office on Drugs concluded that the annual prevalence of cocaine use is estimated at between 0.3 and 0.4 percent of the population aged 15-64. In addition, data showed an increase in the number of cocaine users, from 14 million in 1998 to 18,8 million in 2014 (World Drug Report, 2016).

The known effects of the cocaine can be not only by the action of the drug, but also due to the presence of different contaminants present in it. Until it comes to the final product that is offered to the consumer, the production of cocaine undergoes several processes, receiving different chemical substances. Among the frequent substances encountered, are solvents, alkalizing substances, such as sodium carbonate, organic acids, and oxidizing agents (Oliveira and Wagner, 2013). Another type of adulteration often made is the addition of additives which may be contaminants or diluents, this procedure is performed with the intention of obtaining increased profit generated through sales since it uses less of the pure drug to produce the desired volume (Lapachinske et al., 2015).

Generally, adulterants have pharmacological properties similar to those of cocaine, including lidocaine, caffeine and levamisole. According to data from the Drug Enforcement Administration (DEA), levamisole is present in more than 70% of the seized cocaine in the USA (Tallarida et al., 2014). This medication was previously used as a chemotherapy adjuvant, as well as an

immunomodulator and an anthelmintic drug (Chang et al., 2010), and has been found being the main cause of agranulocytosis and vasculitis in cocaine users (Lee et al., 2012).

Depending upon the route of administration, the absorption of the drug may be faster as in the case of smoked cocaine, or slower, as occurs with the intranasal route, due to the nasal mucosa barrier and the vasoconstriction that is generated. The rate at which cocaine reaches the CNS is also influenced by the route of administration. While endovenous cocaine is diluted in the blood, a large portion of the smoked form is absorbed in the lungs, reaching the brain in a much greater concentration (Cone, 1995). Cocaine also crosses the placental barrier, so children of addicted mothers may be born with withdrawal syndrome since become dependent even on mother's belly. In addition, cardiomyopathies, microcephaly, and behavioral disorders have been observed (Strathearn et al., 2010; Cressman et al., 2012).

The primarily cocaine elimination in organism is by biotransformation. The main metabolites generated is benzoylecgonine (BE), the major metabolite in plasma through the action of carboxylesterase type 1, and ecgonine methyl ester (EME), formed in the liver by cholinesterases and in plasma by butyrylcholinesterase (Goldstein et al., 2009). Also, there is norcocaine, an active metabolite produced from the N-demethylation carried out by enzymes of the cytochrome P-450 family and considered to be a free radical generator and responsible for the impairment of antioxidant defenses (Valente et al., 2012). Initial effects in the organism include intense euphoria, feeling of wellbeing, and great confidence. It can also decrease appetite and sleep. The most common toxic effects of cocaine use include cardiac arrhythmias, seizures, myocardial

infarction, cerebral stroke, and psychiatric disorders, as phobia and depression (Heard et al., 2008; Zhang et al., 2016; Tortajada et al., 2012).

The mechanism of cocaine action in the brain is through the blockade of the dopamine transporters (DAT), leading to an increasing of dopamine availability at the synapse (Volkow et al., 1997). Besides the inhibition of dopamine receiving, it also acts by blocking norepinephrine and serotonin transporters, responsible for the autonomous peripheral effects, such as heart stroke (Ritz et al., 1987). The dopamine excess is eliminated through oxidation and methylation, by the action of monoamine oxidase (MAO) and cathecol-o-methyltransferase (COMT) enzymes, respectively. As a result of its metabolism, occurs the formation of reactive oxygen species (ROS), as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), molecules capable of causing damage to the cells, inducing oxidative stress and finally, apoptosis (Banerjeet al., 2014). The main goal of the present work is to review the literature concerning cocaine toxicity both in vitro and in vivo studies, bringing up an overview of the mechanisms of drug action in different organisms. This review used PubMed database, and the terms used for the search were cocaine, cytotoxicity, *in vitro* and *in vivo*. No inclusion or exclusion criteria were adopted.

1.1 *In Vitro* toxicity

Seizures, hypertension and ischemic stroke have been reported as effects of cocaine in CNS. At the cellular level, signs of apoptosis and necrosis induction were observed. Badisa et al. (2010) demonstrated that cocaine toxicity in glial cells is time and dose-dependent. It was possible to observe morphological differences after 24 and 48 hours between the controls and the

cells treated with cocaine (2-7 mM). Besides being smaller and rounded, the cells also lost its anchoring properties. In the same study, using a fluorometric assay, the group demonstrated a decrease in mitochondrial membrane potential, in a manner that, at a concentration of 4.0 mM, the membrane potential was decreased by 50%.

In agreement, Cunha-Oliveira et al., (2005) demonstrated that acute exposure of rat adrenal phaeochromocytoma cells (PC12 cells) to cocaine 3 mM concentration for 96 hours decreased cell viability approximately in 40%. Corroborating with the idea that cocaine induces apoptosis, Syed et al. (2005) found an increase in the expression of caspase 3 and caspase 9 in PC12 cells treated with cocaine in a dose-dependent manner. Also, Lepsch et al. 2009 demonstrated the induction of apoptosis and necrosis in PC12 cell line treated with cocaine, using markers such as mitochondrial dysfunction, increased LDH release, activation of caspase 3, decrease in Bcl -2 expression and increased cleavage of α- spectrin. Besides signs of apoptosis and necrosis, the cell viability and the mitochondrial activity in presence of cocaine was decreased after 24 h of treatment (Badisa et al., 2012). Lamarche et al., (2017) demonstrated that one of the pathway involved in cocaine toxicity seems to be the opening of mitochondrial permeability transition pore (PTP). PC12 cells exposed to 2 mM of cocaine over 48h were induced to death by PTP opening. Interestingly, when a PTP inhibitor cyclosporin A or metformin was used, the PTP opening was prevented and cell death decreased significantly when compared with the group that had no PTP inhibitor added.

Lepsch et al., (2015) demonstrated in a study using two types of primary cultures, striatal and mesencephalic, the toxic effects of cocaine. In the

striatal culture, after previous treatment, it was possible to observe cell death by apoptosis and inhibition of neurite outgrowth. Also, the primary mesencephalic culture showed signs of apoptotic death and blockade of neurite extension.

The consequences of cocaine exposure during intrauterine life also already have been studied, it is known that cocaine can cross the placental barrier, affecting fetal development. After exposition to cocaine, mouse fetuses neuron was lead to cell death, with induction of apoptosis, observed by biochemical, morphological and functional markers (Nassogne et al., 1997). In addition, neural precursor cells treated with different concentrations of cocaine hydrochloride presented its proliferation inhibited in a dose-dependent manner. A down-regulation of cyclin A, a cell cycle gene that has a role in the entry of cells into S phase, was observed, indicating that cocaine can induce cell cycle arrest. Finally, the cells exposed to cocaine presented changes indicative of differentiation into neurons, which according to the author, it was related to a suppression of SOX2, a transcriptional factor that inhibits cell differentiation (Hu et al., 2007).

The metabolites norcocaine (NCOC) and N-hydroxynorcocaine are seems to be more hepatotoxic than cocaine itself. In this sense, when inhibitors of P450 were administrated before cocaine treatment, liver damage was prevented, proving that the metabolites generated from the oxidation of cocaine are the responsible for the toxic effects (Thompson et al., 1979). The cytotoxicity of cocaine through its oxidative metabolism with ROS generation was demonstrated in previous studies conducted by Zaragoza et al., (2000; 2001). After expose of isolated hepatocytes to increasing doses of cocaine, there was a rise in hydrogen peroxide levels. The authors suggest that the ROS

generation may be an immediate response from cells to cocaine exposure. Besides, the cells showed morphological alterations indicative of apoptosis and necrosis. Mitochondrial superoxide dismutase and catalase activity decreased gradually with the increase in cocaine concentration. All these data corroborate with other studies that demonstrated the link between ROS generation and cellular apoptosis induction in cocaine cell toxicity.

Concerning nephrotoxicity of cocaine, Valente et al., (2012) evaluated the effects of its metabolites such as benzoylecgonine (BE), norcocaine (NCOC), ecgonine (EC), and the methyl ester (EME) in epithelial cells of human proximal tubule primary culture (HPTECs). The metabolism of cocaine in HPTECs leads to the formation of EME and NCOC, but not BE. Norcocaine and cocaine caused a reduction in cell viability in a dose-dependent manner, with NCOC causing significant cell death at a concentration of 2.5 mM. Antioxidant activity of glutathione has been reduced by low doses of cocaine as the mitochondrial is inhibited at high concentrations. It was also described the presence of apoptotic cells even at low concentrations (0.1 to 2.5 mM), whereas necrosis was observed only at high concentrations (5.0 mM).

Cocaine also has an impact on cardiovascular system, blocking the reuptake of norepinephrine and increasing vasoconstriction, as well modulating ion channels (Xiao et al., 1999), leading to an impairment in heart contractility. Besides this, some studies have suggested that cocaine is toxic to myocardial cells altering its structure (Welder et al., 1988; 1993). Myocardial cells exposed to cocaine at different concentrations showed severe LDH leakage and ATP decreasing, indicating cell injury and mitochondrial dysfunction (Yuan and Acosta., 1996). Also, the cytochrome C release from mitochondria into the

cytosol and the activation of caspase 3 and caspase 9 mechanisms were identified after exposure of myocardium cell culture of rat fetuses at 100 uM for 24 hours cocaine, indicating apoptosis. The cells exhibited morphological changes such as shrinkage, rounding, and partial detachment, with condensed and segmented nuclei.

Table 1. Reviewed articles concerning *in vitro* toxicity: author, cell line, dose/concentration of the drug and main toxic effects observed

Author	Cell line/ Type of cell	Dose/Concentration of cocaine	Toxic effects
Badisa et al., 2010; 2012	C6 glial cell	2-7 mM	Morfological changes, decreased mitochondrial membrane potencial.
Oliveira et al., 2005	PC 12 cell	3 mM	Decreased cell viability
Syed et al., 2005	PC 12 cell	50–2500 µM	Increased expression of caspases
Lepsch et al., 2009	PC 12 cell	Not declared	Induction of apoptosis and necrosis, mitochondrial dysfunction, release of LDH, activation of caspases
Lamarche et al., 2017	PC 12 cell	2 mM	PTP mitochondrial opening
Lepsch et al., 2015	Mesencephalic/ Striatal	1 mM	Induction of apoptosis, inhibition of neurite outgrowth and blockade of neurite extension
Nassogne et al., 1997	Cerebrocortical cell	Not declared	Induction of apoptosis of mouses fetuses neurons
Zaragoza et al., 2000; 2001	Hepatocytes	0-1000 µm	ROS production, induction of apoptosis and necrosis, decreased activity of SOD and CAT
Valente et al., 2012	HPTECs	01-10 mM	Reduction of cell viability, reduced activity of GSH, induction of apoptosis and necrosis
Yuan and Acosta., 1996	Cardiomiocytes	10-5 – 10-3 M	LDH leakage and ATP decreased production, induction of apoptosis

1.2 *In Vivo* toxicity

Acute cocaine administration downregulated several transcriptions encoded by the mitochondrial genome, including NADH dehydrogenase and cytochrome C oxidase subunits. Also, after acute cocaine treatment, was observed an increase in the generation of dopaminergic hydroperoxide in areas such as cortex and striatum of male Wistar rats, reaching more than 60% when compared to controls after 12 h of injection (Dietrich et al., 2005).

Using pregnant female rats treated with cocaine, Xiao and Zhang (2008) measured the levels of caspase-3, caspase-8, caspase-9 and the Bax and Bcl-2 protein expression in fetal rat brain. All the results showed a dose-dependent increased activity in caspase-3, caspase-8 and caspase-9 in the fetal brain, a known characteristic of cell death. In the family of Bcl-2 apoptosis genes regulators, Bax acts promoting cell death while Bcl-2 promotes cell survival, inhibiting apoptosis. In the current study, Bax and Bcl-2 expression was dose-dependent increased, suggesting that while is occurring cell death, is activated a protective mechanism in an attempt to balance the cell loss that has occurred.

Many studies already reported the cytotoxic effects of cocaine in hepatic cells. The toxicity caused for the exposition to cocaine goes from release of transaminases into the circulation from mitochondria damage (Gottfried et al., 1986). Male rats treated with acute and binge cocaine showed hepatoxicity and signals of necrosis. Lipid peroxidation was also reported, with an increasing in TBARS levels accompanied by depletion of

ATP and GSH pools. GSH is an important intracellular antioxidant, reacting with the ROS formed, which explain the reduced levels found in the present study (Devi and Chan, 1996).

Some studies have reported the toxicity of cocaine on immune cells. Pacifici et al. (2003) conducted a study with male albino Wistar rats treated with acute and chronic cocaine to verify if the immune-suppression caused by exposure to cocaine could be due the oxidative stress of its oxidized metabolites. The results showed a decrease in natural killer's cells activity and in the production of Th1-type cytokine IL-2 (related to lymphocytes proliferation), whereas Th2-type cytokine IL-10 presented an increase in its production. It was also observed that the proliferation of lymphocytes was already suppressed with a single administration. A recent research demonstrated an increased in the activity of NF-κB, a transcription factor involved in immune-inflammatory responses, in cerebellum of rats treated with cocaine for 18 days. Also, there was an increment in ED1 expression, a protein present in inflammatory development expressed by macrophage and microglia. However, the antioxidant responses level was decreased, which, according with the author, could be related with the increase in glutamate concentrations that was observed (López-Pedrajas et al., 2015).

Regarding pulmonary toxicity, severe complications due cocaine abuse has been reported being influenced by the administration route in a dose dependent manner. Fibrosis, asthma and interstitial pneumonitis have been associated with the use of the smoked form (Restrepo et alt., 2007). After systemic administration of cocaine in male rats, progressive pulmonary lesions were observed in different cellular structures, including multiples hemorrhages

and increase of fibrous connective tissue. Also, the walls of veins and arteries appear shrunken and inflammatory infiltrates were found, these findings linking cocaine use and lung toxicity may be correlated with redox cycling (Barroso-Moguel et al., 1999).

Different studies have shown that cocaine can also cause DNA damage by the formation of ROS. A study conducted with female rats treated with repeated cocaine doses showed that occurred DNA damage in different brain areas (De Souza et al., 2014).

Using male rats, Alvarenga et al. (2010) also demonstrated that cocaine can cause genotoxic damage in cell blood and brain. Liver also showed DNA damage, according to Yujra et al. (2015), that using male mice treated acutely with crack cocaine verified genetic damage in hepathocytes only at the highest dose, and there was also genetic damage in brain and peripheral cells. Parolini et al. (2017) using a zebrafish embryo model exposed to cocaine, benzoylecgonine (BE) and ecgonine methyl ester (EME) observed a decrease in cell viability. The authors also observed DNA fragmentation and induction of apoptosis in all concentrations of cocaine, BE and EME, and necrosis when exposed to the highest concentration of the drugs. The rise in antioxidants defenses activity demonstrated an increase in ROS levels.

In humans, the consequence of cocaine renal toxicity is associated with complications as nephrotic syndrome, acute glomerulonephritis and rhabdomyolysis (Jaffe and Kimmel, 2006). In addition, an *in vivo* study of nephropathy due cocaine chronic administration, with 28 male rats received cocaine hydrochloride 5 days per week at a dosage of 30 mg.kg/day, showed animals with progressive renal lesions in the glomerular capillary endothelial

cells, and Bowman's capsule epithelial cells, as well as lesions of wall cells in the tubule and interstitium. Glomerulus showed loss of their original architecture and at day 90, glomerulus was smaller, showed deformities, altered capillaries and was adhered in part to the Bowman's capsule, moreover, was observed fibroblasts and fibrin in the capsular space. After 30 days, necrosis of some of the tubular cells in distal collecting tubules (DCT) and proximal collecting tubules (PCT) was observed. These findings were accompanied by nuclear karyorrhexis or pyknosis, cytoplasmic eosinophilia and vacuolization (Barroso-Moguel et al., 1995).

Cocaine use is closely linked to the induction of cardiovascular and cerebrovascular diseases such as vasoconstriction, hypertension, myocardial infarction, contractility reduction, and ventricular dysfunction (Bachi, 2017). A recent study demonstrated the induction of oxidative stress in myocardium of individuals with suspected death following the use of large amounts of cocaine. When compared to the control group, the expression of oxidative and nitrosative stress markers, such as i-NOS, 8-OHdG and NOX2, was increased. Liou et al., (2014) demonstrated that after chronic exposure of male Wistar rats to cocaine, the drug induced apoptosis in cardiomyocytes by activation of the cardiac Fas receptor-dependent apoptotic pathway and mitochondrial-dependent apoptotic pathway. In addition, the Fas and mitochondrial-dependent apoptotic pathways appeared to be more marked in the study group than in the control group, which shows, according to the authors, the evident relationship between oxidative stress and cocaine cardiotoxicity (Turillazzi, 2017). The Fas–FasLigand complex is an effector of cell death that may be induced by oxidative stress, leading to activation of caspases (Bishopric et al., 2001).

Table 2. Reviewed articles concerning *in vivo* toxicity: author, animal model, dose/concentration

Author	Animal model	Dose/ Concentraion of cocaine	Toxic effects
Dietrich et al., 2005	Rats	20 mg/kg	Downregulation of transcptions encoded by mitochondrial genome, generation of dopaminergic hydroperoxide
Xiao and Zhang, 2008	Rats	30 and 60 mg/kg	Increased caspases and Bax/Bcl-2 genes activity
Devi and Chan, 1996	Rats	25 and 40 mg/kg	Induction of necrosis, lipidic peroxidation and depletion of GSH activity
Pacifici et al., 2003	Rats	40 mg/kg	Decreased IL-2 production, increased IL-10 production, supression of lymphocytes proliferation
Lopes-Pedrajas et al., 2015	Rats	15 mg/kg	Increased NF-KB activity, decreased antioxidants activity, expression of ED1
Barroso-Moguel et al., 1999	Rats	30 mg/kg	Pulmonary lesions
De Souza et al., 2014	Rats	15 mg/kg	Genotoxic damage
Alvarenga et al., 2014	Rats	1-7 mg/kg	Gnotoxic damage
Yujra et al., 2015	Mice	0-18 mg/kg	Genotoxic damage
Parolini et al., 2017	Zebrafish	0.004-40 nM	DNA damage, induction of apoptosis and necrosis, increased antioxidants enzymes activity and ROS levels
Barroso-Moguel et al., 1995	Rats	30 mg/kg	Renal lesions, induction of tubular necrosis
Liou et al., 2014	Rats	10 mg/kg	Induction of apoptosis via Fas pathway.

1.3 Conclusion

In a general way, cocaine appears to have a very similar mechanism of toxicity in the different organisms and cells studied. We summarize in Figure 1 the possible effects of cocaine in the cellular context. The formation of free radicals is either by the metabolism of catecholamines or the drug itself plays a key role in the generation of oxidative stress and, consequently, induction of genotoxic damage of cell death with activation of caspases and alteration in mitochondrial membrane potential. Cocaine can increase the levels of ROS, due its consequence, the expression of antioxidants enzymes is increased by activation of NF-kB. Nevertheless, when the levels of ROS are too high to the amount of enzymes available, the depletion of antioxidants defenses induce oxidative stress. This unbalance redox status besides causing lipidic peroxidation of membranes, also can leads to activation of the extrinsic and intrinsic apoptotic pathways. Both extrinsic, characterized by the ligation of Fas-Ligand to Fas receptor or TNF-α to TNF-Receptor, and intrinsic via, release of citocrome c from mitochondria to cytosol by expression of pro-apoptotic enzymes, lead to activation of caspases. Due the instability of mitochondrial membrane, this organelle also acts as source of reactive oxygen species, which could induce DNA demage, another import factor of cell death induction.

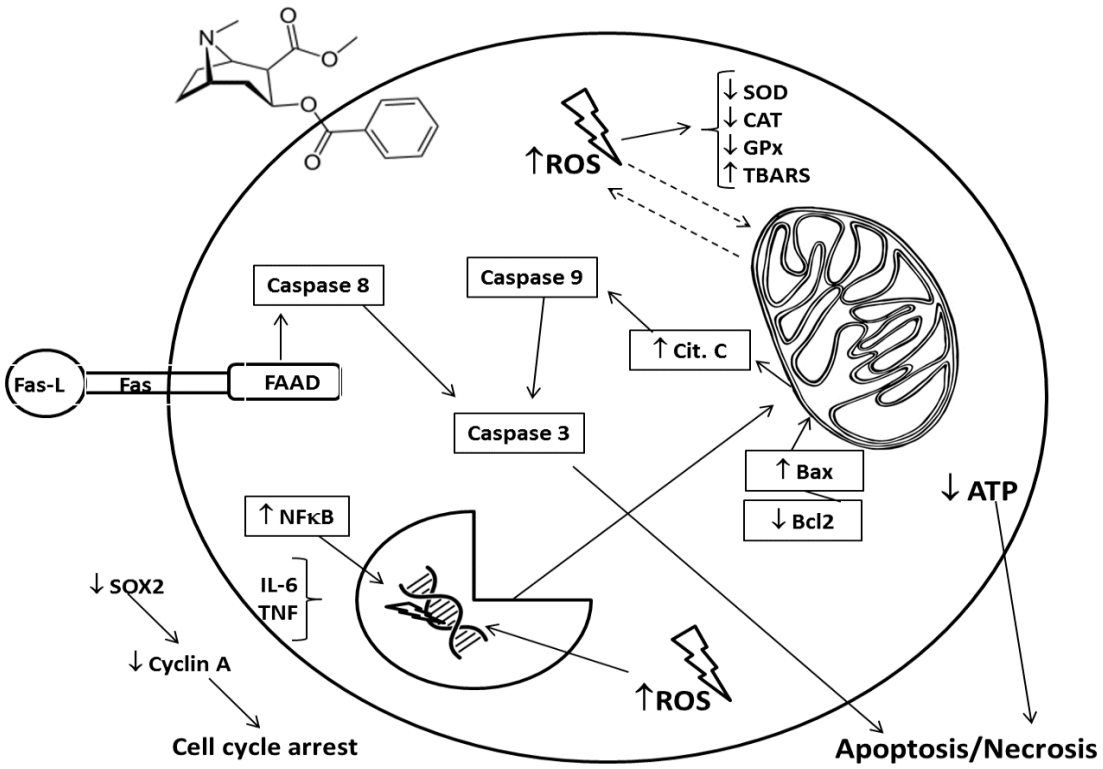

Figure 1. Scheme of cell death pathways induced by exposure of cells to cocaine.

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In vitro model to study cocaine and its contaminants

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Abstract

Cocaine is one of the most popular illicit drug worldwide. Due its great addictive potential, which leads to euphoria and hyperactivity, it is considered a public health concern. At the central nervous system, the drug acts inhibiting catecholamine re-uptake. It is now known that in addition to the toxicity of the drug itself, the contaminants present in the street drug have raised concern about the harmful effects on health. Toxicological *in vivo* and *in vitro* studies have demonstrated the toxic effects of cocaine correlated with the generation of reactive oxygen species, which in turn lead to oxidative damage to the cells. Therefore, the aim of this study was to propose an *in vitro* model using an immortalized culture of glioblastoma to study the action mechanisms of cocaine at cellular level and role of its adulterants associated to the toxicity. For that, were evaluated cell death and oxygen reactive species induction, oxidation of macromolecules as membrane lipids and DNA, and loss of mitochondrial membrane potential after cocaine exposure. The results showed that cocaine can decrease cellular viability in a dose-dependent way after exposure to cocaine over 24 and 72 h. These results were observed both in the immortalized and primary culture, C6 cell and astrocytes, respectively. Cocaine also induced cellular death in a way that, at the highest concentration, more than 50% of the cells were in apoptosis. However, in the seized drug, the predominant cell death was due to necrosis. Using dichlorofluorescein (DCF) assay, we confirmed reactive oxygen species production after increasing concentrations of cocaine exposition over 24 h. In agreement with these findings, occurred an increasing in MDA production, as well as increased superoxide dismutase (SOD) and catalase (CAT) activity. The induction of DNA

damage was observed after exposure to all cocaine concentrations, both in the alkaline and modified version. Our results demonstrate the occurrence of mitochondrial dysfunction by depolarization of mitochondrial membrane as a consequence of cocaine exposure. In summary, these results demonstrated that cocaine can induce reactive oxygen species formation, leading to oxidative stress. As a consequence of this unbalance, DNA damage, lipidic peroxidation and loss of mitochondrial membrane occurred, wich could be an answer to cell death observed.

Keywords: Cocaine, C6 rat glioma, Damage,

Abbreviations: CAT: Catalase, CNS: Central nervous system, DCFH-DA: 2',7'-dichlorofluorescein diacetate, DMEM: Dulbecco's modified Eagle medium, DMSO: Dimethilsulfoxide, DNA: Deoxyribonocleic acid, FBS: Fetal bovine serum, MAO: Monoamineoxidase, MDA: Malondialdehyde, MPT: Mitochondrial permeability transition, MTT: (3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide, ROS: Reactive oxygen species, SOD: Superoxide dismutase, TB: Trypan blue, TBARS: Thiobarbituric acid reactive substances

1. Introduction

Cocaine is one of the most commonly used drugs in the world, with an estimated 18 million users worldwide (World Drug Report, 2016). Due to the high rate of morbidity and mortality, its use is considered a growing social and public health problem. In the United States, it affects about 2% of the population between 15-64 years, while in Europe it has a lifetime prevalence between 0.8 and 0.9% of the adult population (World Drug Report, 2016).

The most common symptoms related to acute use are tachycardia, hypertension, euphoria, feelings of well-being and, subsequently, cardiac and respiratory depression (Gay et al., 1975). In addition, myocardial ischemia and stroke have also been observed in drug users (Jaffe J.A and Kimmel P.L., 2006). As a result of the elimination of excess dopamine, by auto-oxidation or by the action of the enzyme monoamine oxidase (MAO), occurs the generation of reactive oxygen species (ROS) (Hermida-Ameijeiras et al., 2004).

Several *in vivo* and *in vitro* studies have related the toxic potential of cocaine with the formation of ROS, thus inducing oxidative stress (Uys et al., 2011; 2014; Valente et al., 2012). The generation of nitrogen peroxide and superoxide anion has already been investigated as a cause of liver damage induction (Vitcheva V., 2012). Among the causes of cardiotoxicity of cocaine, it is mentioned the generation of aminochrome through the metabolization of catecholamines excess. These molecules, when passing through the redox cycle in the mitochondria, lead to the formation of free radicals (Varga et al., 2015). Studies have demonstrated the induction of apoptosis in myocardial cells (Zhang et al., 1999) and increased cytosolic cytochrome C levels. In an animal model, maternal exposure of rats to cocaine led to increased caspase activity in

the fetal heart and deregulation of the levels of Bax and Bcl-2 proteins, linked to apoptosis mediated by mitochondrial signaling (Xiao et al., 2000). Although the exact mechanisms of cocaine neurotoxicity induction have not yet been fully elucidated, evidence has correlated the induction of oxidative stress by the drug and the observed CNS damage. After exposure to cocaine, the reduction of antioxidant defenses and induction of cell death by apoptosis has been observed in previous studies (Lopes-Pedrajas et al., 2015; Dietrich et al., 2005; Nassogne et al., 1997) as well as inhibition of neurite outgrowth (Marshall, 2002) and genotoxic damage (Alvarenga et al., 2009; de Souza et al., 2014).

In addition to the toxic effects associated with cocaine use mentioned earlier, another growing concern is related to the adulterants added to the drug. Various adulterants are added to cocaine in order to increase the volume of the drug and thereby increasing the profits related to its illicit market. Studies reporting forensic analysis on the composition of cocaine seized in Brazil revealed that the most common adulterants are caffeine, lidocaine, procaine, benzocaine, phenacetin, diltiazen and more recently levamisole (Marcelo et al., 2015; Floriani et al., 2014; Magalhães et al., 2013; Bernardo et al., 2003; Mídio et al., 2000), These substances can have serious adverse health consequences or even cause premature death (Cole et al., 2010). In this sense, analytical methods dedicated to analyze simultaneously cocaine and its main adulterants are required to gain an accurate picture of adulteration. Additionally, biological models predicting the toxicity of adulterated drugs are also important to provide a vigilance of these substances and can provide decrease of risks to public health.

Due to the impact of cocaine use related to health aspects, associated with the absence of a model that explains in a global way the effects of this drug, this work aimed to evaluate an *in vitro* model to access cocaine toxicity, which can be used to infer about a possible toxic effect of associated adulterants.

2. Materials and Methods

2.1. Chemicals

DMEM (Dulbecco's modified Eagle medium), FBS (fetal bovine serum), trypsin–EDTA, L-glutamine, penicillin/streptomycin and TB (trypan blue) were obtained from GIBCO (Grand Island, NY, USA). The H_2O_2 (hydrogen peroxide), MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide) and DCFH-DA (2',7'-dichlorofluorescein diacetate) were purchased from Sigma (St. Louis, MO, USA). Low melting point agarose and agarose were obtained from Invitrogen (Carlsbad, CA, USA). Annexin V-Phycoerythrin (PE) and 7-Amino-Actinomycin (7-AAD) were purchased from BD Biosciences (San Diego, CA). Standart cocaine hydrochloride (0.1 mg/ml; Enila, Brazil) and one seized sample of cocaine hydrochloride (n° registration: 21222016; Federal Police of Brazil) were prepared in fresh Phosphate Buffer Solution (PBS) as a stock solution of 0.1 M and kept at 4°C. Cocaine working solutions, ranging from 2 mM to 15 mM, were prepared immediately before use by diluting the stock solution with PBS. All others reagents were analytical grade.

2.2. Cell Culture and Treatment

C6 rat glioma cell line was bought from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were grown in DMEM supplemented with 5% FBS at 37°C in a humid atmosphere containing 5% of CO_2 and harvested by treatment with 0.15% trypsin–0.08% EDTA in PBS. Cells ($5x10^5$ cells) were seeded in complete media and grown for one day prior to treatment with the cocaine before evaluation of cell profile by determination of viability, death type, DNA damage and redox status.

In addition, primary astrocyte cultures were used as a non-transformed cell model in order to evaluate the cocaine toxicity and were prepared as previously described (Da Frota et al., 2009). Briefly, cortex of newborn Wistar rats (1–2-day-old ones) were removed and mechanically dissociated in a Ca^{+2} and Mg^{+2} free balanced salt solution, pH 7.6, (137 mM NaCl, 5.36 mM KCl, 0.27 mM Na_2HPO_4, 1.1 mM KH_2PO_4 and 6.1 mM glucose). After centrifugation at 1000 rpm for 5 min, the pellet was resuspended in DMEM supplemented with 10% FBS. The cells ($5x10^5$) were plated in poly-l-lysine-coated 48-well plates. After 4 h plating, plates were gently shaken and medium was then replaced to remove neuron and microglia contaminants. Cultures were allowed to grow to confluence for 20–25 days. Medium was replaced every 4 days. These cells were treated with cocaine for 24 h and survival, ROS production and mitochondrial function were evaluated.

2.3. Cell Viability Assay

Tetrazolium dye MTT reduction was performed according to Denizot and Lang, 1986. Briefly, after the treatments, cells were washed once with PBS

before the addition of 100 µL of serum-free media containing yellow tetrazolium salt (MTT; 1 mg/mL) dye. After 3 h of incubation at 37°C, the supernatant was removed and the residual purple formazan product was solubilized in 200 µL DMSO, stirred for 15 min, and its absorbance was measured in a SpectraMax reader M2e (Molecular Devices, USA) at a wavelength of 570 nm. The absorbance of the negative control was set as 100% viability, and the values for treated cells were calculated as a percentage of the control. Besides that, trypan blue dye-exclusion assay (TBDE) was used to measure cell viability. For each treatment, 10 µL of cell suspension was mixed with 10 µL of 0.4% trypan-blue solution. Cytotoxicity (the cellular growth inhibitory rate) was determined from the number of viable cells (no color) in treated samples as a percentage of the PBS control. We used the Countess®Automated Cell Counter (Invitrogen, California, United States). The test was carried out according to the instructions of the manufacturer.

2.4. Apoptosis Induction

Annexin V-PE was used with a vital dye, 7-AAD, to distinguish apoptotic (Annexin V-PE positive, 7-AAD negative) from necrotic (Annexin V-PE positive, 7-AAD positive) cells. After treatment, cells were trypsinized, collected and resuspended in 40 µL of binding buffer with 2 µL Annexin V-PE. Cells were incubated for 15 min in the dark at room temperature. After incubation, 160 µL of binding buffer and 2 µL of 7-AAD were added. Cells were incubated for 5 min and additional 200 µL of binding buffer were added. Data were collected and analyzed by a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with CellQuest software, in a total of 10,000 events per sample;

fluorescence was measured and the percentage of viable, apoptotic and necrotic cells was determined.

2.5. Comet Assay

The comet assay was performed as reported by Singh et al, 1988. Cell culture (~ 10^4 cells/mL) was mixed with low melting point agarose solution and spread on agarose-precoated microscope slides. For each treatment, three slides were made of the well. Slides were incubated in ice-cold lysis solution (2.5 mol/L NaCl, 10 mmol/L Tris, 100 mmol/L EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4°C for at least 1 h to remove cell membranes. Then, slides were placed in a horizontal electrophoresis unit and incubated with fresh alkaline buffer solution (300 mmol/L NaOH, 1 mmol/L EDTA, pH 13.0) at 4°C for 15 min to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was conducted for 15 min at 25 V (94 V/cm). In the modified comet assay, slides were removed from the lysis solution and treated with DNA repair enzymes, they were subjected to electrophoresis as previously described. The slides were washed for 5 minutes each in enzyme buffer (40mM HEPES-KOH, 1M KCl, 5mMEDTA, 2.5mg/mL bovine serumalbumin fraction V-BSA, and pH 8.0). The suspension was added to the slide, covered with coverslip, and incubated for 45 (ENDO III) and 30 minutes (FPG) at 37∘C. All these steps were performed under yellow light or in the dark to prevent additional DNA damage. Slides were stained using silver nitrate. One hundred cells from each treatment were selected and analyzed for DNA migration and the average of the three slides from each treatment group was used to determine the damage index. The damage index is an arbitrary score calculated for cells in different damage classes, which are scored visually according to the

tail length of the “comet” into five classes: Class 0, undamaged, without a tail; Class 1, with a tail shorter than the diameter of the head nucleus; Class 2, with a tail length one- to twofold greater than the diameter of the head; Class 3, with a tail longer than twofold the diameter of the head; and Class 4, comets with no heads. The damage index ranges from 0 (no tail) to 400 (maximum migration).

2.6. Measurement of lipid peroxidation

The lipid peroxidation was determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product formed by lipid peroxidation. The assays were performed according to Salgo and Pryor (1996), with minor modifications. As TBA reacts with other products of lipid peroxidation in addition to MDA, results are expressed in terms of TBARS, which was determined by absorbance at 532 nm by spectrophotometry. Hydrolyzed tetramethoxypropane (TMP) was used as the standard. The results were normalized by protein content.

2.7. Reactive Oxygen Species Formation

Levels of intracellular ROS were estimated following treatment with cocaine using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) as a fluorescent probe. Detection of oxidative stress was done by incubating the cells with 20 μM of H_2DCFDA for 20 min at 37°C. Cells were then detached by trypsinization and washed twice with PBS. Cells were analyzed using a FACS Calibur flow cytometer with CellQuest software. A total of 10,000 events were measured per sample. DCF fluorescence intensity was shown in arbitrary units.

2.8. Superoxide dismutase (SOD) and catalase (CAT) activity

SOD activity was evaluated by inhibition of superoxide-dependent autoxidation of epinephrine, verifying the absorbance of the samples at 480 nm (Misra e Fridovich, 1972). One SOD unit is defined as the amount of SOD necessary to inhibit 50% of epinephrine autoxidation and the specific activity is reported as SOD Units/mg protein.

CAT activity was assayed according to the method described by Aebi (1984), based on the disappearance of H_2O_2 at 240 nm. One CAT unit is defined as 1.0 µmole of hydrogen peroxide consumed per minute and the specific activity is calculated as the number of CAT Units/mg protein.

2.9. Mitochondrial Transmembrane Potential

Mitochondrial transmembrane potential was determined by flow cytometry using rhodamine 123 dye retention. Briefly, cells were washed with PBS, incubated with rhodamine 123 (1ug/mL) at 37°C for 15 min in the dark and washed twice. Cells were incubated again in PBS at 37°C for 30 min in the dark, and fluorescence was measured by cytometry. Ten thousand events were evaluated per experiment and cellular debris was omitted from the analysis.

2.10. Seized cocaine analysis by Gas chromatography coupled to mass spectrometry (GC-MS) and Total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) method

Both GC-MS and ATR-FTIR analyses were performed by the Technical and Scientific Division of the Braziliam Federal Police. For GC-MS analyses, an

Agilent 6890 N gas chromatograph interfaced with an Agilent 5973 mass selective detector (Agilent Technologies, Palo Alto, CA, USA) was employed. Separation was performed in a 30 m × 0.25 mm × 0.25 μm 5% phenylmethylpolysiloxane column (HP-5MS, Agilent J&W Scientific, Folsom, CA,USA). Injection was carried out in split mode. Helium (99.999% purity) was used as carrier gas. Data were acquired in the full scan mode (50–500 m/z mass range). The total ion chromatograms (TIC) were integrated. Compounds found in samples were identified using mass spectrum computerized databases (NIST02), data coming from literature and analytical standard for cocaine and their main adulterants.

Using the ATR-FTIR method, the infrared spectra were obtained using a Nicolet 380 FTIR Spectrometer (Nicolet Instrument Co., Madison, USA) equipped with DTGS (deuterated triglycine sulphate) detector and smart orbit single reflection diamond ATR sampling accessory was employed for all experiments. The absorbance was measured in the wavelength range of 1800–650 cm^{-1} whereas the resolution was 4 cm^{-1}. Thirty two scans were performed for each sample.

2.11. Statistics

All experiments were repeated at least three times. Data were expressed as means ± standard error of the mean (SEM) values. Statistical analyses of the data were performed using ANOVA One-Way, and the means were compared using Tukey's multiple comparison test. *P*-values less than 0.05 were considered to be significant.

3. Results

3.1. Cocaine-induced cell death

With the purpose of evaluating different exposures we performed treatment of 24 h, mimetizing an acute exposure, and of 72 h, mimetizing a chronic exposure and evaluated the cellular viability by MTT assay. Our results showed that in both times of treatment cocaine induces C6 cell decrease viability, in a dose-dependent way in all concentration (Fig. 1A).

Due to the possible limitations of the MTT method we also evaluated cytotoxicity using the trypan blue exclusion assay and the results were similar showed a IC_{50} of 6.76 mM after 24 h of cocaine exposure (Supplementary Fig. 1). To check if the cytotoxic effect is the same in a primary culture we used rat astrocytes and assessed viability by trypan blue. Interestingly our results showed that primary culture have the same cytotoxicity profile presented by immortal culture C6 cells (Fig. 1B), suggesting that the *in vitro* model is suitable for cocaine cytotoxicity assessment.

As we did not observe a difference between the IC_{50} after 24 and 72 h, we chose to follow our evaluations using the 24 h treatment. In the next step we evaluated the type of death induced by cocaine treatment, our results shows that after 24 h of cocaine exposure a remarkable decrease was observed in the viable cells population (unlabeled with either annexin-V or 7-AAD) compared with the untreated control cells (Figure 1C). In the highest concentration (15 mM), approximately 53% of the cells were in apoptosis and 21% in necrosis, while the control group presented 12% in late apoptosis and 6% in necrosis,

approximately. These results corroborate with cell viability assays and indicate that the major form of death is by apoptosis.

3.2. Damage to macromolecules

In order to determine the damage triggered by cocaine in C6 cells, we examined the degree of DNA damage and lipid peroxidation induced by cocaine exposure. The levels of DNA damage were analyzed by alkaline and modified comet assay with Endonuclease III (ENDO III) and Formamidopyrimidine DNA glycosylase (FPG) enzymes, after cocaine exposure. Figure 2A shows an increase in the levels of DNA index damage in all concentrations when compared with negative control group. The lesion specific repair enzymes FPG and Endo III were used to increase the sensitivity of the assay. When compared with the group without enzymes, occurred an amplification of the breaks in all concentrations. As seen in Figure 2B, the treatment of cells with cocaine resulted in a dose-dependent increase in TBARS production. This increase was statistically significant after exposure at concentrations of 10 and 15 mM ($P < 0.05$; ***$P < 0.001$).

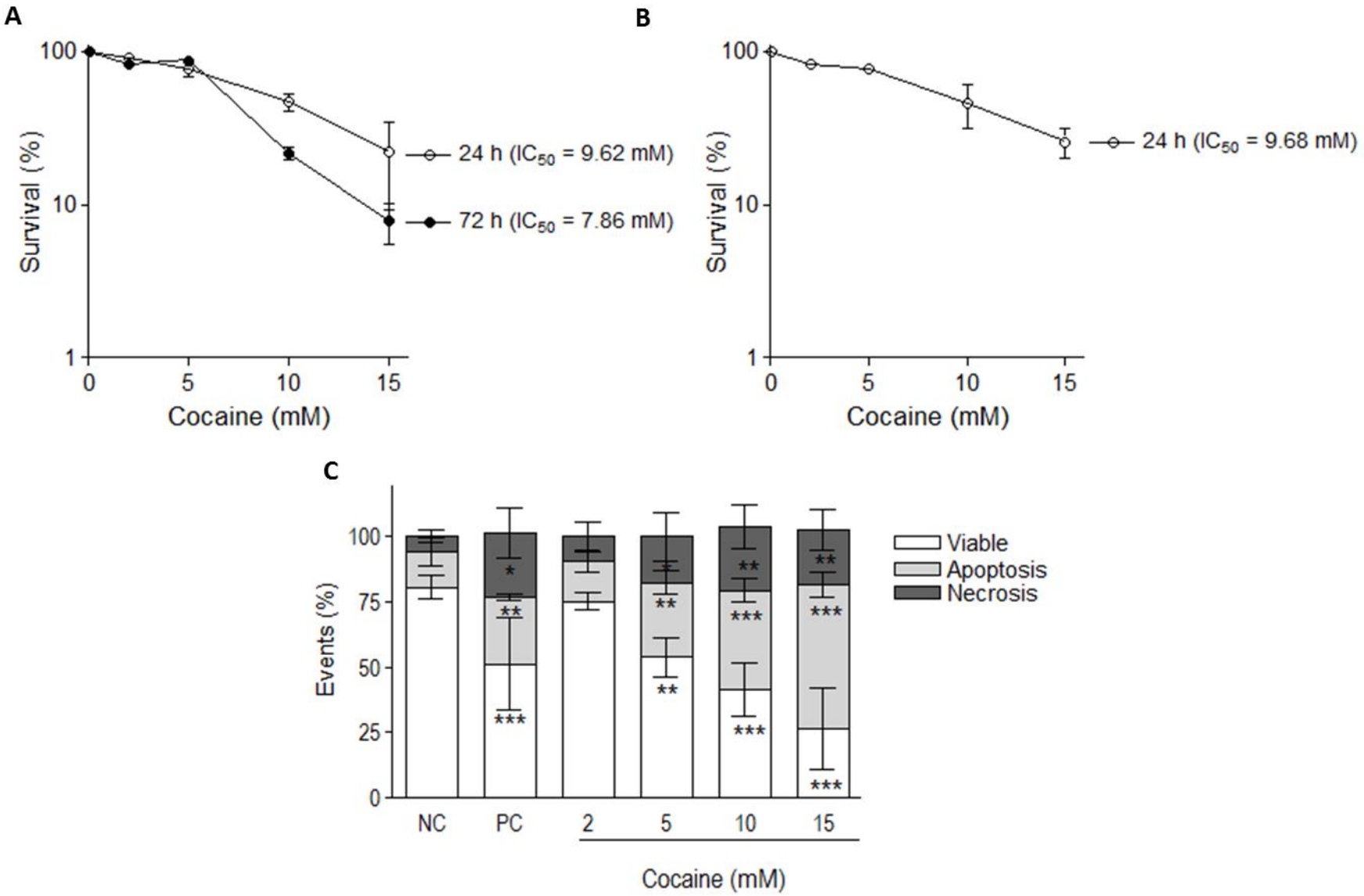

A
100
10
1
Survival (%)
0
5
10
15
Cocaine (mM)
24 h (IC50 = 9.62 mM)
72 h (IC50 = 7.86 mM)
B
100
10
1
Survival (%)
0
5
10
15
Cocaine (mM)
24 h (IC50 = 9.68 mM)
C
100
75
50
25
0
Events (%)
NC
PC
2
5
10
15
Cocaine (mM)
Viable
Apoptosis
Necrosis
*
**
***

Fig. 1. Cocaine treatment over 24 h induced cell death by apoptosis/necrosis. **A**) Cell viability of C6 cells after cocaine exposure for 24 h and 72 h; **B**) Cell viability of astrocyte cells after 24 h incubation; **C)** Cocaine treatment induces apoptosis in C6 cells after treatment for 24 h with. In the survival assays the results were expressed as total percentage survival with mean ± SEM. The sum of the percentages of Annexin V and 7-AAD-PE-positive cells was calculated. At least three independent experiments were pooled and analyzed as a combined data set. Results are expressed as means ± standard error (SEM). *Significant difference as compared to negative control treatment at $P < 0.05$; **$P < 0.01$; ***$P < 0.001$/One-way ANOVA Tukey's multiple comparison test. H_2O_2 was used as positive control (PC) and induced an increase in cell death.

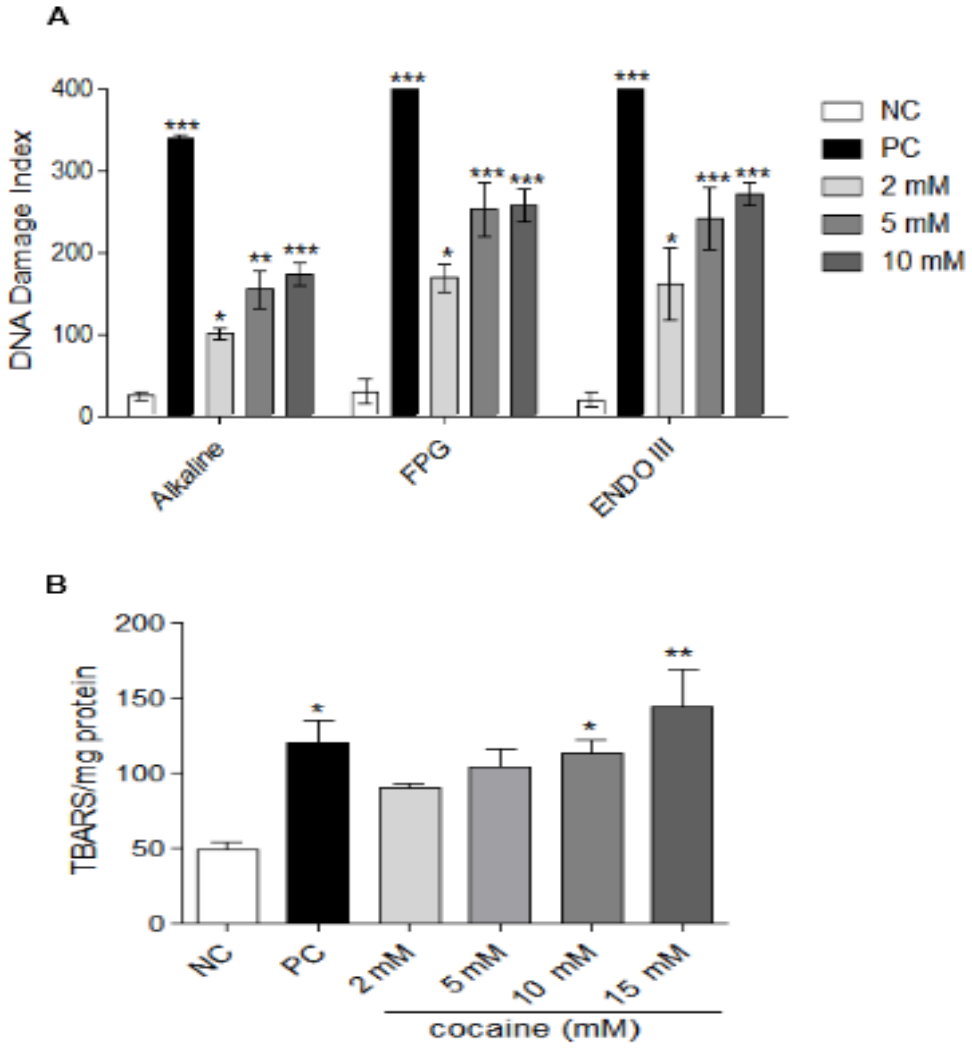
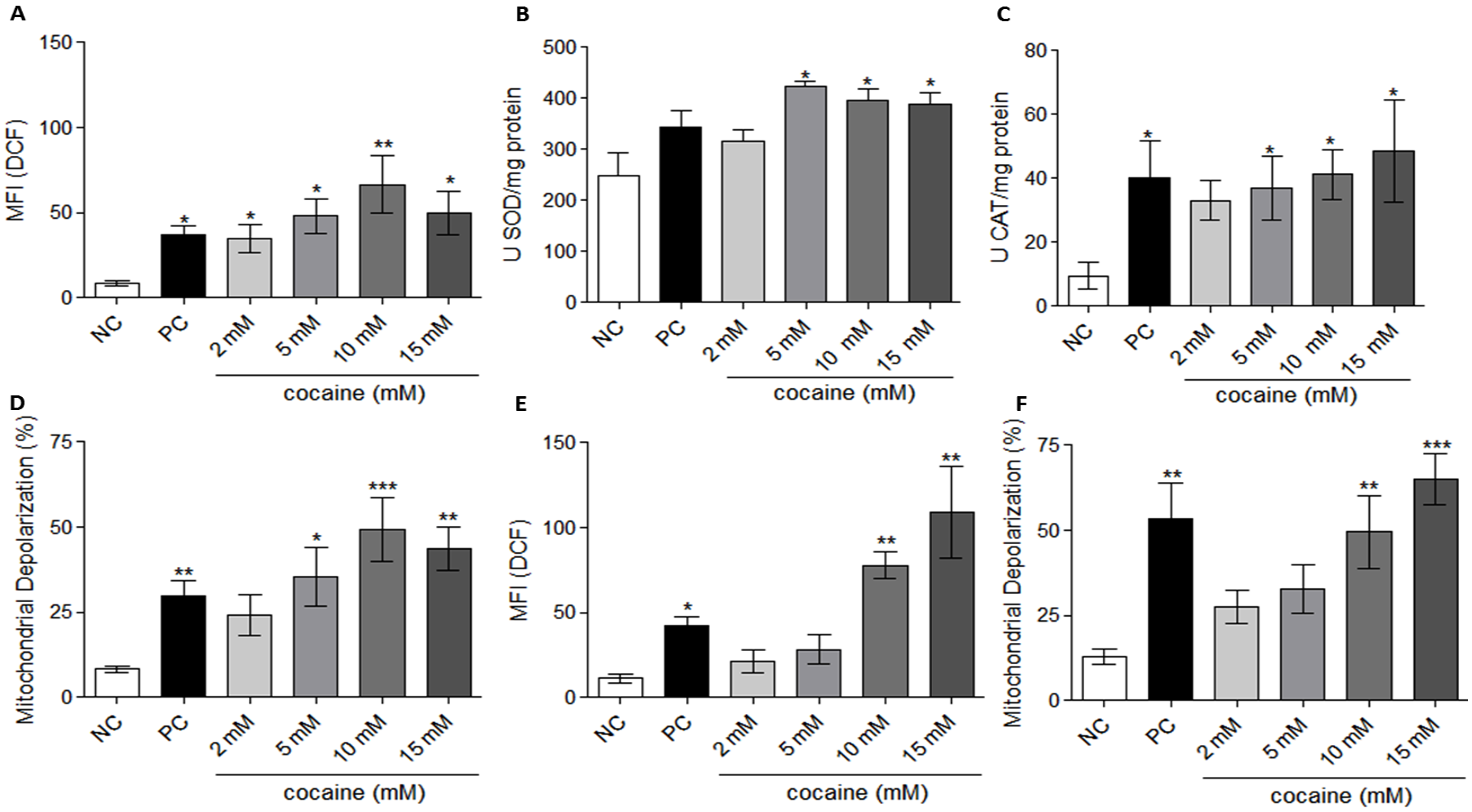


Fig. 2. Cocaine induces macromolecules damage in C6 cell after 24 h of exposure. **A**) Induction of DNA strand breaks by cocaine as evaluated by the comet assay in alkaline conditions, without and with DNA damage repair enzymes FPG and ENDO III treatment; **B**) Determination of TBARS in cells treated with cocaine at the indicated concentrations. Bars represent the mean ± standard error (SEM) of three independent experiments. *Significant difference as compared to negative control treatment at $P < 0.05$; ***$P < 0.001$/One-way ANOVA Tukey's multiple comparison test. H_2O_2, used as positive control (PC), induced a higher level of DNA damage (in alkaline comet and after treatment with FPG and ENDO III) and lipid peroxidation (black bars).

3.3. Redox status

It is known that cocaine leads to an increase of reactive species. In order to evaluate if cell death and macromolecule damage are associated with radical species, we evaluated the redox profile of the cells treated with cocaine. Our results demonstrated that cocaine induces an increase in the DCF mean fluorescence intensity in all concentrations, indicating the ROS formation after cocaine exposure (Fig. 3A). The alteration in redox profile induced by cocaine was confirmed by increase in SOD (Fig. 3B) and CAT (Fig. 3C) activities, which were significantly elevated by cocaine in comparison with negative control. These results could be explained by the acute exposure, besides that, by the time of enzyme activity quantification, the cells defenses still could not have been exhausted. These results can be associated with the increase in alteration of mitochondrial membrane potential. The involvement of mitochondria in the formation of reactive species was also evaluated by the measurement of mitochondrial depolarization and our results showed an increase in depolarization induced by cocaine indicating a mitochondrial dysfunction (Fig. 3D). The formation of reactive species and the mitochondrial dysfunction induced by cocaine are also observed in primary astrocytes (Fig. 3E and 3F).


A
MFI (DCF)
150
100
50
0
NC
PC
2 mM
5 mM
10 mM
15 mM
cocaine (mM)
B
U SOD/mg protein
500
400
300
200
100
0
NC
PC
2 mM
5 mM
10 mM
15 mM
cocaine (mM)
C
U CAT/mg protein
80
60
40
20
0
NC
PC
2 mM
5 mM
10 mM
15 mM
cocaine (mM)
D
Mitochondrial Depolarization (%)
75
50
25
0
NC
PC
2 mM
5 mM
10 mM
15 mM
cocaine (mM)
E
MFI (DCF)
150
100
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0
NC
PC
2 mM
5 mM
10 mM
15 mM
cocaine (mM)
F
Mitochondrial Depolarization (%)
75
50
25
0
NC
PC
2 mM
5 mM
10 mM
15 mM
cocaine (mM)

Fig. 3. Redox status of cells after 24 h of cocaine treatment. In C6 cells: **A**) Media of DCF fluorescence intensity; **B**) Measured of SOD antioxidant enzyme activity; **C**) Measured of CAT antioxidant enzyme activity; **D**) Measured of mitochondrial depolarization. In primary astrocyte cells: **E**) Media of DCF fluorescence intensity; **F**) Measured of mitochondrial depolarization. Results are presented as mean and standard error median of at least three independent experiments. Data significant in relation to negative control group at $^{**}P < 0.05$; $^{***}P < 0.01$; $^{***}P < 0.001$/One-way ANOVA Tukey's multiple comparison test. H_2O_2, used as positive control (PC), induced an increase in DCF oxidation, SOD and CAT activity (black bars).

3.4. Real sample of seized cocaine qualitative analysis and its cytotoxicity evaluation

After determination of the standard cocaine toxicity model, our next step includes the evaluation of one sample of cocaine seized by the Brazilian Federal Police, in Caxias do Sul city, Rio Grande do Sul State. The qualitative analysis of this sample was made by the Scientific Technical Division. The drug was in the form of granules and powder, with white color, crystalline and brilliant in appearance and characteristic odor. The analyses showed a cocaine signal obtained at 13.07 minutes and levamisole at 9.98 minutes, using GC-MS analyses (Fig. 4). Also, the mass spectrum of the sample identified the presence of levamisole as a contaminant.

Interestingly, we observed a cytotoxicity profile for cocaine seized (Fig. 5A) rather similar to standard drug (Fig. 1A) after 24 and 72 h of treatment, showed a IC_{50} of 8,2 mM and 6,9 mM, respectively. However, when we evaluated the type of cell death, in despite of the same cytotoxicity profile, we found an increase in death due to necrosis to cocaine seizure (Fig. 5B). Comparison of percentages of death by necrosis induced by standard cocaine and cocaine seized indicates a significant difference between drugs (5C).

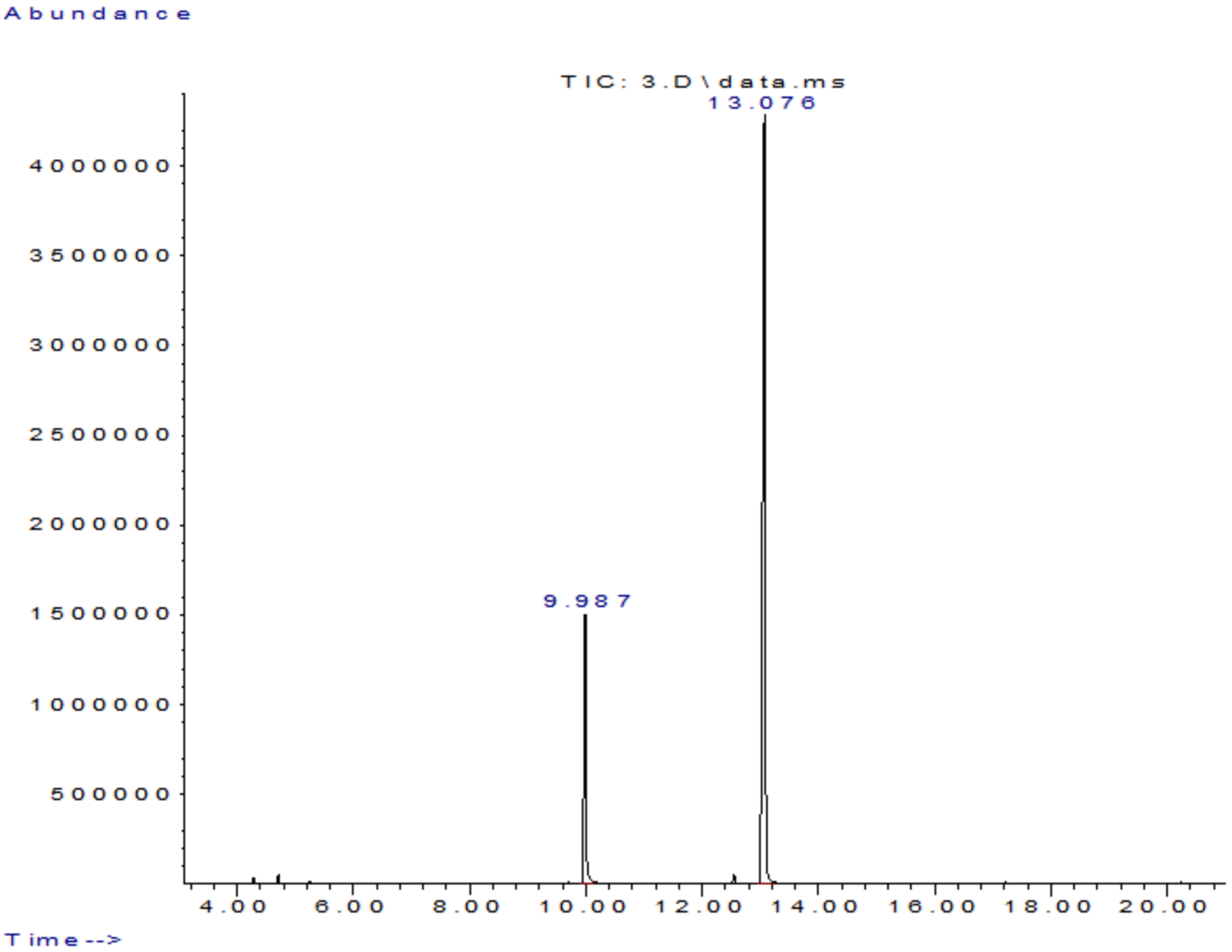
Abundance
TIC: 3.D\data.ms
13.076
9.987
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Time-->

Fig. 4. Cocaine qualitative analysis. Chromatogram of total ions obtained for a questioned sample (Sample 3), with cocaine signal in 13.07 minutes and levamisol signal in 9.98 minutes

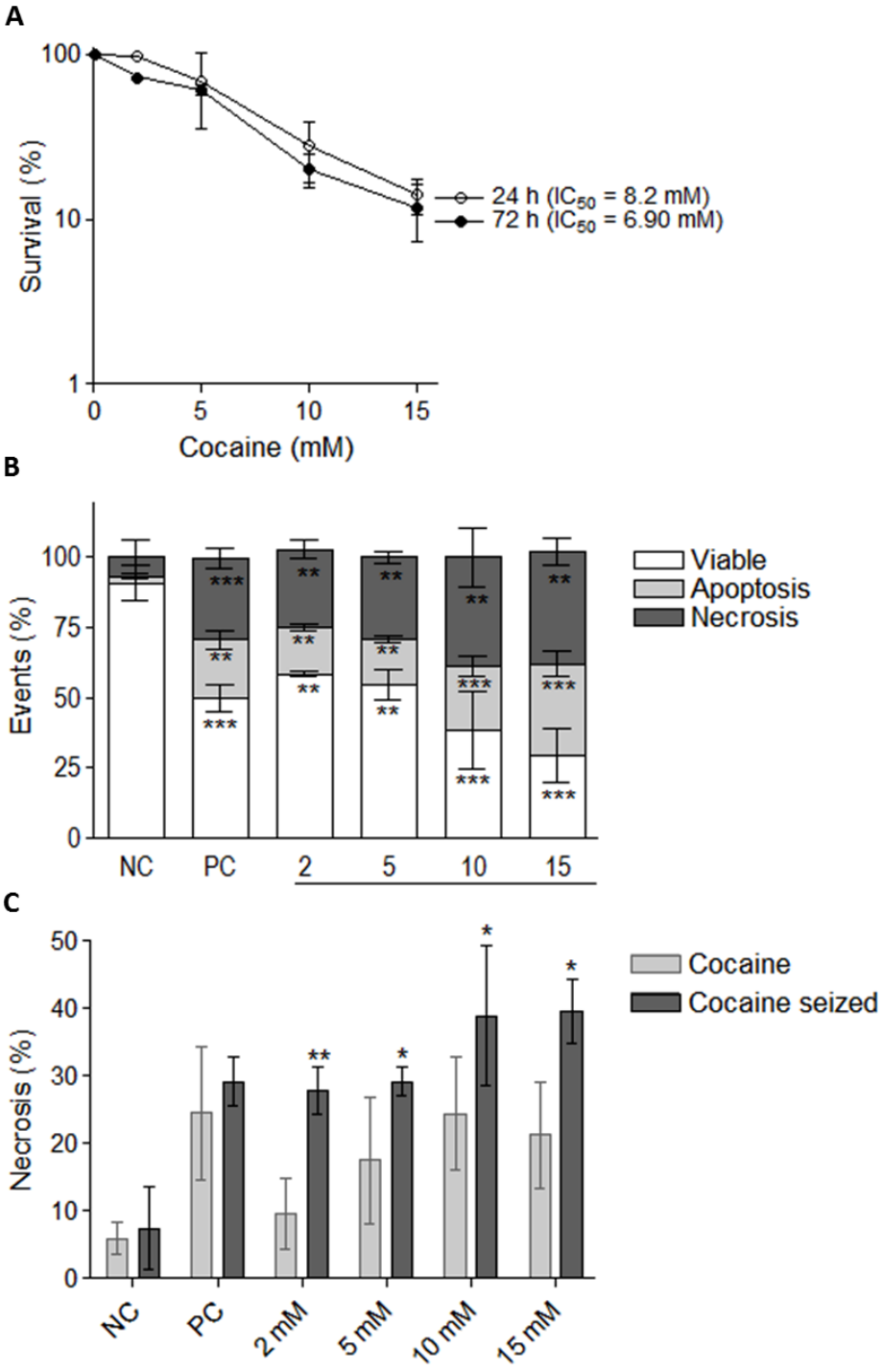

Fig. 5. Cocaine seizure cytotoxicity. **A**) Cell viability of C6 cells using MTT reduction assay after cocaine seized exposure for 24 h and 72 h; **B**) Cocaine seized treatment induces necrosis in C6 cells. **C**) Comparison of percentage of

cells in necrosis after treatment with standard cocaine or cocaine seized. In the survival assays the results were expressed as total percentage survival with mean ± SEM. The sum of the percentages of Annexin V and 7-AAD-PE-positive cells was calculated. At least three independent experiments were pooled and analyzed as a combined data set. Results are expressed as means ± standard error (SEM). *Significant difference as compared to negative control treatment at $P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$/One-way ANOVA Tukey's multiple comparison test. H_2O_2 was used as positive control (PC) and induced an increase in cell death.

4. Discussion

Cocaine toxicity at CNS level is related to interaction with dopamine transporters, increasing the level of the neurotransmitter at the synaptic cleft and generating a great amount of reactive species (Pereira, 2015). Previous studies had demonstrated cocaine toxic effects in different *in vitro* and *in vivo* models, therefore the aim of this work was to propose an *in vitro* model that reunites the main parameters of toxicity of the drug already observed in the literature so far. For this, we used the C6 glial cells, also, to check the efficiency of our *in vitro* model we also used astrocyte primary culture.

Our model was efficient to demonstrate the decrease of cocaine-induced cellular viability in a dose-dependent way. In addition, we have shown that these cells are mostly dying by apoptosis. Cocaine abuse can lead to toxic effects, including causing damage in specific brain areas. Studies in humans, animals, and cell cultures, have shown the toxic effects of cocaine, which can lead to cell death (Lepsch et al., 2015). In agreement with previous study of Badisa et al., 2010, we also observed under a light microscope, change in the

cellular morphology. After treatment with cocaine, the cells became rounded and grouped, with formation of intercellular gaps (Supplementary Fig. 2).

Many studies have shown that cocaine induces cell death by apoptosis (Oliveira M.R. and Jardim F.R., 2016; Cerretani et al., 2012). Liou et al. (2014) demonstrated that a greater activated cardiac Fas- and mitochondria-dependent apoptotic pathway after chronic cocaine exposure might provide one possible mechanism to explain the development of heart failure in chronic cocaine users. Apoptosis is a programmed cell death that plays a fundamental role in the maintenance of cellular homeostasis and has as characteristics, the decrease of cell volume, chromatin condensation and nuclear fragmentation (Pérez-Garijo A. and Steller H., 2014; Kawamoto et al., 2016). Our results appoint that exposure to cocaine induces loss of mitochondrial membrane potential and damage to macromolecules, such as DNA and lipids, therefore this could explain the apoptotic cell death observed.

One of the mechanisms involved in the neurotoxicity of cocaine is associated with excess dopamine at the synaptic terminals. Studies have shown that dopamine auto oxidation leads to the generation of ROS, a fact that culminate in the induction of oxidative stress (Yamato, 2010). In the present study, using the DCF assay, we demonstrate that the exposure to increasing concentrations of cocaine led to the formation of ROS in treated glioblastoma cells for 24 h. Banki et al. (1999), showed that an increase in the levels of ROS production was linked to mitochondrial membrane hyperpolarization and these facts resulted in Fas-mediated cell death.

During mitochondrial oxidative phosphorylation, the electrons released from reduced substrates are transferred to oxygen molecules and it is during

this process that the production of ROS occurs, such as the superoxide anion, for example, that can interact with nitric oxide, also producing reactive nitrogen species (Marchi et al., 2012). The hyperpolarization of the mitochondrial membrane leads to mitochondrial permeability transition (MPT) pore opening, and as a result occurs the release of reactive species into the cytoplasm. This mechanism, called Reactive Oxygen Species (ROS)-induced ROS release (RIRR) seems to act as a signal to open the channels in the others cell mitochondria, a fact that could induce loss of mitochondrial function and cell death (Zorov, 2014). Our study demonstrates that cocaine exposure causes a significant mitochondrial membrane potential loss. These findings could be explained by the oxidative stress that the cell was exposed after reactive species generation, leading to mitochondrial reactive species release.

Therefore, the model of the C6 cell *in vitro* evaluation of cocaine toxicity is in accordance with the literature data. Since our results show that cocaine induces cytotoxicity, probably by an increase of ROS formation which can cause genotoxic damage and oxidation of membrane lipids. The increase in antioxidant enzymes activity observed in this work could be due the acute exposure conducted. It is possible that, by the time of enzyme activity quantification, the cell response to the generated stress was still active, and the enzyme system defense had not yet been exhausted. the latter, as a defense mechanism against the high level of ROS. Furthermore, the exposition to cocaine induced loss of mitochondrial membrane potential, which can be the cause or consequence of the increase in reactive species. All these factors justify cell death by apoptosis.

Surprisingly, when we tested our in vitro model against the seized cocaine, although we observed the same cytotoxicity profile, the cell death results indicate that the seized cocaine induced more necrotic death than the standard cocaine. The type of induced cell death difference observed could be explained by the presence of adulterants in the seized sample, in this case, the levamisole confirmed by the qualitative analysis performed. Previous studies have demonstrated that levamisole-adulterated cocaine can induce skin necrotic lesions, also agranulocytosis and neutropenia (Le garff, 2016; Lawrence, 2014; Larocque, 2012). Although the action mechanism of this anthelmintic has not been fully elucidated, it is believed that it may be associated with inhibition of norepinephrine reuptake, and during the course of its metabolism the formation of aminorex, an amphetamine-like metabolite, which would explain his choice as a drug adulterant (Freyer, 2012).

5. Conclusion

Considering the severity associated with the adulterants in cocaine seized, this model appears suitable for a preliminary evaluation of the toxicity of the seized drug sample associated with the analytical assays of the contaminants. Thus, based on the results obtained, the use of this *in vitro* model to evaluate the cocaine toxicity is considered a reliable alternative to provide subsidies for drug characterization and understanding of effects and symptoms observed in drug users.

Conflict of interest statement

The authors have no competing interests.

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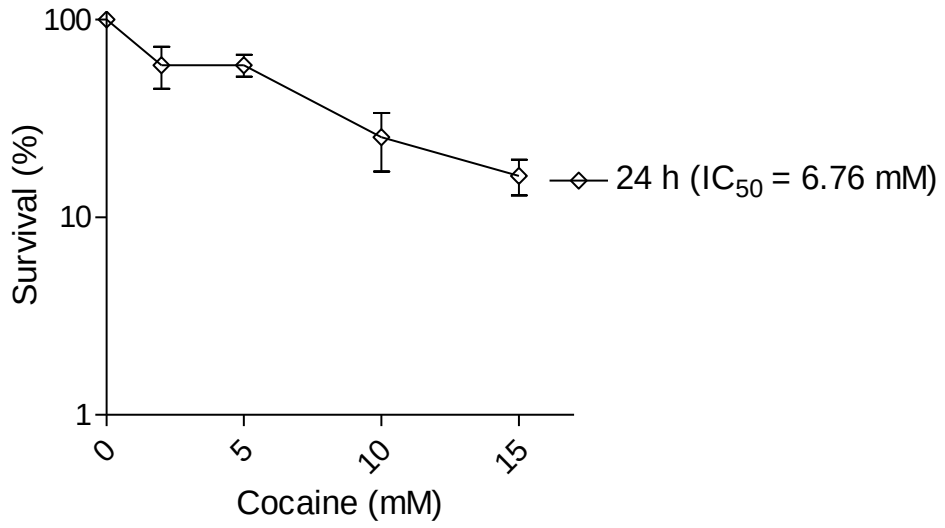
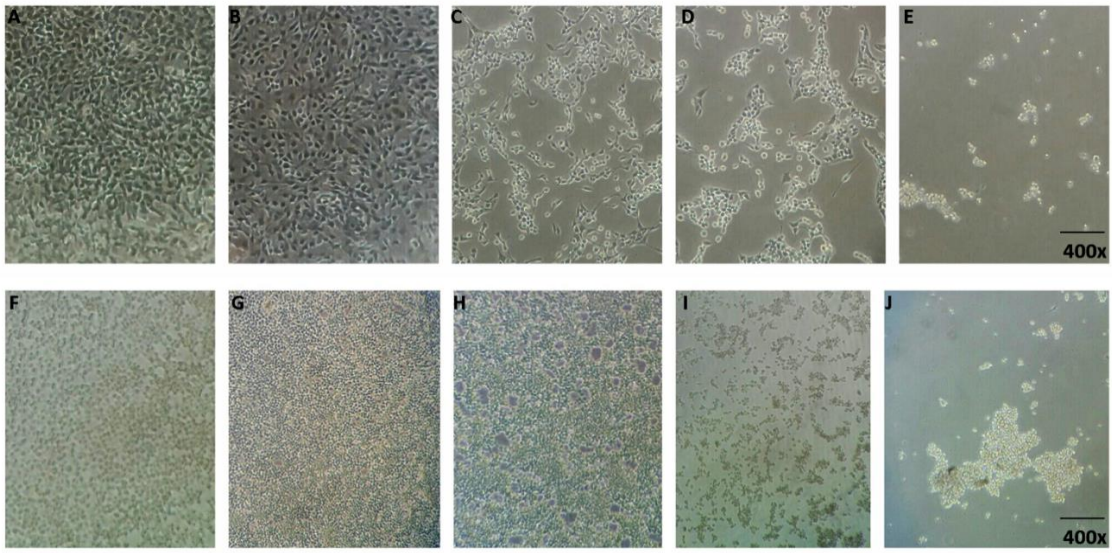
Supplementary Fig. 1



Supplementary Fig. 1. Cell viability of C6 cells using trypan blue exclusion assay after cocaine exposure for 24 h. The results were expressed as total percentage survival with mean ± standard error of mean (SEM). At least three independent experiments were pooled and analyzed as a combined data set.

Supplementary Fig. 2



Supplementary Fig. 2. Morphology of C6 cells treated as cocaine for 24h. Standard Cocaine: **A**) Negative Control; **B**) 2mM; **C**) 5mM; **D**) 10 mM; **E**) 15 mM. Cocaine seized: **F**) Negative Control; **G**) 2mM; **H**) 5mM; **I**) 10 mM; **J**) 15 mM. Images were collected using an optical microscope with an increase of 400X.

6. Discussão Geral

O abuso de drogas é uma das maiores preocupações relacionadas à saúde pública mundial, fato que acaba elevando as taxas de mortalidade e morbidade, aumentando os encargos com os cuidados à saúde (Sanchéz-Hervás, 2016). O uso de cocaína tem sido associado a diversas patologias observadas tanto em usuários da droga quanto em modelos *in vivo* e *in vitro*, afetando principalmente os sistemas cardiovascular, respiratório e nervoso central. Decorrente do uso crônico de cocaína, a nível de sistema nervoso central, cita-se o aumento nos riscos de infarto, depressão, convulsões e, nos casos mais graves, morte (Liao et al., 2016). Além da toxicidade da droga em si, muitos trabalhos têm relacionado a nefrotoxicidade da cocaína com os contaminantes encontrados nela, em especial, o levamisol, capaz de induzir danos neste órgão (Mionuddin et al., 2016; Carraca et al., 2016).

Conforme a revisão bibliográfica realizada, o mecanismo de ação da cocaína parece ser bastante semelhante nos diferentes órgãos e modelos estudados. Alguns trabalhos demonstraram que após exposição a doses crescentes de cocaína por um período de 24h ou mais, ocorreu a redução da viabilidade celular de uma forma dose dependente. Também foi observada a ativação de caspases 3 e 9, as quais desempenham importante papel na ativação de morte celular por apoptose, descrito em diferentes trabalhos aqui apresentados. Além da indução de morte celular por apoptose, também há ocorrência de necrose nas doses mais elevadas da droga. A atividade mitocondrial parece comprometida devido a exposição à cocaína. Uma causa comum aos danos observados nestes estudos parece ser o estresse oxidativo

induzido pela formação de espécies reativas de oxigênio. Estudos realizados para medir os níveis de defesas antioxidantes apontaram que ocorre uma diminuição de enzimas como GSH e SOD, com o aumento na concentração de cocaína. Tal fato pode ser explicado pela superprodução de ROS nas doses mais elevadas da droga, o que consequentemente esgotaria a capacidade natural do organismo de eliminá-los.

Neste estudo foram avaliados os mecanismos de toxicidade da cocaína em linhagem celular C6 de glioblastoma, e em células de cultura primária de astrócitos. Por meio dos ensaios de viabilidade celular por MTT e azul de tripan, podemos observar uma redução na sobrevivência celular de maneira dose dependente após 24 e 72h de exposição avaliado por MTT (IC_{50}: 9,62 e 7,86, respectivamente) e 24h no ensaio com azul de tripan. Na análise realizada com microscópio óptico observamos uma notória diferença na morfologia das células após a exposição a doses crescentes da cocaína quando comparadas com o grupo controle, de forma que nas doses mais altas (10 e 15mM) as células tornaram-se arredondadas e agrupadas, formando grande espaço intercelular.

Podemos observar que a cocaína induziu morte celular, predominantemente por apoptose em todas as concentrações, com uma diferença significante entre o número de células viáveis existentes no grupo controle negativo e as existentes nas maiores concentrações. Por outro lado, quando as células foram expostas à droga de apreensão, o tipo de morte prevalente nas maiores concentrações foi necrose. A partir da análise do perfil químico da droga realizada pela Polícia Federal, acreditamos que a morte celular por necrose possa estar intimamente ligada aos contaminantes

presentes neste tipo de droga. Alguns trabalhos relativos a este tema, já evidenciaram a correlação entre as manifestações clínicas observadas em usuários da droga, como agranulocitose, lesões púrpuras na face, glomerulonefrite e hemorragia nos pulmões, e a presença de concentrações elevadas de levamisol na cocaína utilizada (Berman et al., 2016; Nolan et al., 2015).

A administração de cocaína induz a perda do potencial de membrana mitocondrial levando a liberação de espécies reativas de oxigênio (Devi, 1997; Masini, 1997). Acredita-se que em determinados tecidos como coração e cérebro, a mitocôndria seja a principal fonte de ROS no organismo (Jezek, 2005). Após 24h de tratamento, por meio do ensaio com Rodamina-123, contatamos a indução de hiperpolarização da membrana mitocondrial, o que pode ser consequência do estresse oxidativo gerado pela droga. Sendo assim, além das espécies reativas de oxigênio geradas pela cocaína, a própria mitocôndria celular pode ter sido fonte desses radicais livres, devido a sua desestabilização de membrana. No presente estudo, após exposição das células durante 24h à cocaína, verificamos o aumento dos níveis de ROS em resposta a todas as concentrações de tratamento quando comparadas com o grupo controle. O estresse oxidativo induzido pelo aumento dos níveis de ROS foi confirmado por meio do aumento na atividade enzimas antioxidantes SOD, responsável pela neutralização de ânions superóxidos, convertidos em peróxido de hidrogênio, e CAT, a qual converte a molécula de peróxido de hidrogênio em água. Outra consequência dos elevados níveis de ROS, foi a peroxidação lipídica observada, indicada pelo aumento nas taxas de formação de malondialdeído.

7. Conclusões

A partir dos dados obtidos durante este estudo, pode-se concluir que:

- A viabilidade celular foi reduzida de forma dose dependente após exposição das células por 24 e 72h à cocaína;

- Tratamento das células com cocaína padrão e cocaína de apreensão induz morte celular por apoptose e necrose, sendo que, a morte por necrose foi maior nas células tratadas com a droga apreendida;

- A cocaína induz a formação de espécies reativas de oxigênio e a disfunção mitocondrial, bem como aumento na atividade das enzimas antioxidantes SOD e CAT.

- Há ocorrência de danos ao DNA e de peroxidação lipídica induzidos pela exposição à cocaína;

8. Perspectivas

- ❖ Utilizar o modelo de toxicidade trabalhado no presente estudo para avaliar a toxicidade de outras amostras de cocaínas de apreendidas;

- ❖ Avaliar a toxicidade da cocaína e de seus contaminantes em um modelo *in vivo.*

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