UNIVERSIDADE FEDERAL DE SÃO PAULO

INSTITUTO DE SAÚDE E SOCIEDADE

PROGRAMA DE PÓS-GRADUAÇÃO INTERDISCIPLINAR EM CIÊNCIAS DA SAÚDE

DANIEL VITOR DE SOUZA

ATIVIDADE INFLAMATÓRIA, OXIDANTE E PROLIFERATIVA EM FÍGADO E RIM DE RATOS EXPOSTOS AO CRACK

S A N T O S

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Dissertação apresentada ao Programa de Pós Graduação Interdisciplinar em Ciências da Saúde da Universidade Federal de São Paulo – Campus Baixada Santista como requisito para obtenção do título de Mestre em Ciências.
Orientador: Prof. Dr.º Daniel Araki Ribeiro
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S A N T O S

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LISTA DE ABREVIATURAS E SIGLAS

® – Marca registrada;
º C – Grau Celsius;
8-OHdG – 8-hidroxi-2'-desoxiguanosina;
8-OHGua – 8-Hidroxiguanina;
ABC – Complexo Avidina-Biotina;
ANOVA – Análise de Variância;
ATP – Adenosinatrifosfato;
CDK – Quinases dependentes de ciclinas;
CEDEME – Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia;
CEUA – Comissão de Ética no Uso de Animais;
cm – Centímetros;
CRP – Citocromo P_{450} redutase (CRP);
CYP450 – Família citocromo P450;
DAB – 3,3-diaminobenzidina;
DAMP – Padrão Molecular Associados a Danos Celulares;
DP – Desvio padrão;
DMSO – Dimetilsulfóxido;
DL50 – Dose letal mediana;
DNA – Ácido desoxirribonucleico;
EDTA – Ácido etilenodiamino tetra-acético;
ELISA – Ensaio de Imunoabsorção Enzimático;
ERO's – Espécies reativas de oxigênio;
EUA – Estados Unidos da América;
FADM – Flavina-adenina dinucleotídeo monooxigenases;
GSH – Glutationa reduzida;
H_2O_2 – Peróxido de hidrogênio;
H_2O – Água;
H_2SO_4 – ácido sulfúrico;
H.E – Hematoxilina-Eosina;
HCL – Ácido clorídrico;
HIV – Vírus da Imunodeficiência Humana;
HO• – Radical hidroxila;
IKK – Complexo de quinases de IκB;
IL-1β – Interleucina 1 subunidade beta;
IL-6 – Interleucina 6;
IL-10 – Interleucina 10;
i.p– Intraperitoneal;
IRAK – Receptor de interleucina;
LPS – Lipopolissacarídeos;
mL – Mililitro;
mtDNA – DNA mitocondrial;
MyD88 – Fator de diferenciação mieloide 88;
nDNA – DNA nuclear;
NF-κB – Fator de Transcrição Nuclear *kappa* B;
nm – Nanômetro;
O_2 – Oxigênio;
$O_2^{\bullet -}$ – Ânion-radial superóxido;

PAMP – Padrão Molecular Associado a Patógeno;
PBS – Tampão fosfato-salino;
PMSF – Fluoreto de fenilmetilsulfonil;
pH – Potencial Hidrogeniônico;
RIP – Proteína de interação do receptor de quinases;
RPM – Rotações por minuto;
SOD – Superóxido Dismutase;
TAB – Proteína ligante de TAK;
TAK – Fator de crescimento β associado à quinase;
TIRAP – Molécula adaptadora associada a receptores do Tipo Toll;
TNF-α – Fator de necrose tumoral subunidade alfa;
TRAF-6 – Receptor associado ao fator de necrose tumoral do tipo 6;
TRIF – Adaptador indutor de interferon-β;
TRL – Receptores do Tipo Toll;
μm – Micrômetro;
UNIFESP – Universidade Federal de São Paulo;
μL – microlitro;
-COOH – Radical ácido carboxílico;
-NH_2 – Radical amino;
-OH – Radical hidroxilo;
-SH – Radical sulfidrila.

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RESUMO

O crack é uma das drogas ilícitas mais utilizadas ao redor do mundo, sendo o consumo e a disseminação desenfreada representam grande problema de saúde pública. O objetivo do presente estudo foi investigar os efeitos da exposição subaguda de crack no contexto inflamatório, oxidativo e proliferativo em fígado e rim de ratos Wistar. Para tanto, o trabalho foi dividido em 4 capítulos a citar: o Capítulo I foi dedicado a estabelecer uma revisão de literatura acerca do assunto; o Capítulo II apresentamos uma revisão de literatura intitulada "Genotoxicity, oxidative stress and inflammatory response induced by crack cocaine: relevance to carcingoenesis" publicada na Revista Environmental Science and Pollution Research; o Capítulo III apresentamos o artigo intitulado "Histopathological and inflammatory response in multiple organs of rats exposed to crack", que foi aceito para a publicação na Revista International Journal of Environmental Health Research e finalmente o Capítulo IV apresentamos o artigo intitulado "Genomic instability suppresses toll like signaling pathway in rat liver exposed to crack cocaine, que foi aceito para publicação na revista In Vivo.

Palavras-chave: crack; citotoxicidade; imunossupressão; rim; fígado

ABSTRACT

Crack cocaine is one of the most widely used illicit drugs worldwide, with consumption and widespread dissemination seen as a major public health problem. The aim of the present study was to investigate the effects of subacute crack cocaine exposure in the inflammatory, oxidative and proliferative context in the liver and kidney of male Wistar rats. To this end, the study was categorized into 4 chapters as follows: Chapter I was dedicated to establishing a literature review on the subject; Chapter II presents a literature review entitled “Genotoxicity, oxidative stress and inflammatory response induced by crack cocaine: relevance to carcinogenesis ”, in which was published to Environmental Science and Pollution Research; Chapter III we present the article entitled “Histopathological and inflammatory response in multiple organs of rats exposed to crack“, in which was accepted into International Journal of Environmental Health Research finally chapter IV we present the article entitled “Genomic instability suppresses toll like signaling pathway in rat liver exposed to crack cocaine, which was accepted into In Vivo.

Key-words: crack cocaine; cytotoxicity; immunosuppression; kidney; liver

Dedico este trabalho aos meus pais e minha irmã, por todo carinho, incentivo, apoio e compreensão durante essa jornada que escolhi trilhar. Amo vocês!

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"*Eu quero aprender mais, compreender mais, evoluir sempre. Quero ter direito a sonhar alto e alcançar meus objetivos com garra e determinação. Quero conquistar novos conhecimentos, todos os dias. E fazer de cada dia uma lição de vida. Quero desenvolver os meus talentos e alcançar os meus objetivos, sem deixar de olhar para o mundo ao meu redor. Quero encarar cada desafio como oportunidade. E fazer dos obstáculos minha maior motivação. Eu sei o que quero. Eu quero é vencer*."

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1. CONSIDERAÇÕES GERAIS

A cocaína é uma substância de origem vegetal, com propriedades anestésicas bem conhecidas. A extração da cocaína ocorre a partir das folhas de coca (*Erythroxylum coca*, gênero *Erythroxylum* da família *Erythroxylaceae*), arbusto de origem sul-americana (Ribeiro & Laranjeira, 2012; Ferreira & Martini, 2001). Entretanto, os produtos formulados à base de cocaína são considerados drogas ilícitas (crack, cloridrato de cocaína, merla), haja visto possuem efeitos estimulantes do sistema nervoso central (CEBRID, 2012). O crack é das formas de apresentação da cocaína, porém a mais deletéria, devido a presença de resquícios de outros agentes químicos (adulterantes) (Passagli, 2013; Riezzo *et al.*, 2012). Quimicamente, essa droga apresenta-se sob a forma de base com aspecto de cristais de coloração marrom, e/ou bege amarelado (Andrade Filho *et al.*, 2013; Oliveira, 2017).

Nos últimos anos, a utilização e a disseminação desenfreada do crack no Brasil vêm sendo encaradas como um grande problema de saúde pública, devido à grave dependência dos seus usuários (Horta *et al.*, 2018; Blanco *et al*., 2015). Em razão disso, comportamentos de riscos e atos de violência são observados. Tais condutas afetam a sociedade como um todo, colocando em risco a saúde e a segurança da população geral (Chaves *et al.*, 2011).

Há ainda uma alta prevalência de atendimentos nos serviços de saúde de consumidores dessa droga ao redor do mundo. Nos Estados Unidos da América (EUA), por exemplo, o crack é responsável pelo maior número de atendimentos nos serviços de urgência e emergência acometido pelo uso de drogas ilícitas (Andrade Filho *et al.*, 2013). No Brasil, a situação parece caminhar na mesma direção, sendo cada vez mais frequente o atendimento nesses serviços devido ao uso indiscriminado do crack (Andrade Filho *et al*., 2013; OBID, 2005). É importante destacar que, o consumo de drogas a base de cocaína, parece ser um fator importante na diminuição da expectativa de vida, em especial, de usuários que possuem co-morbidades, tais

como portadores do vírus da hepatite C (HCV) e do vírus da imunodeficiência adquirida (HIV) (Campa *et al.*, 2016).

Os indivíduos que possuem o hábito de consumir certas drogas, sejam elas lícitas ou ilícitas, buscam na grande parte dos casos, um efeito psicoativo recreativo. Contudo, tais ações culminam com uso indiscriminado dessas substâncias, em decorrência da grande probabilidade de se estabelecer uma dependência física ou mesmo psicológica, afetando a qualidade de vida do indivíduo (Rang *et al.*, 2016). Tais consequências são ainda mais preocupantes quando se trata do uso abusivo de drogas ilícitas, cuja fabricação é realizada de forma caseira, sem qualquer controle, tornando-a uma substância suspeita no que tange aos riscos à saúde (Fukushima *et al.*, 2019).

Estudos recentes vêm demonstrando que drogas a base de cocaína seriam capazes de gerar estresse oxidativo em diversos órgãos, resultando em uma superprodução de espécies reativas de2013). Os tipos de lesões bem como a frequência com que elas ocorrem devido ao con oxigênio (Narvaez *et al.*, 2013). Tal processo possui um papel importante na patogênese de diversas enfermidades (Narvaez *et al*., sumo dessa droga, variam de acordo com a dose e susceptibilidade individual, assim como, diferentes tecidos podem apresentar respostas biologicas distintas (Yujra *et al.*, 2015).

Diante dessas consequências, pesquisadores acreditam que o crack, poderia ter um efeito deletério mais potente quando comparado ao cloridrato de cocaína, visto que o consumo do mesmo estaria associado a diversas lesões teciduais, podendo gerar efeitos sistêmicos ao organismo (Freitas *et al*., 2014).

Entretanto, não é conhecido até o presente, os mecanismos moleculares pelo quais o crack exerce sua toxicidade em múltiplos órgãos, principalmente, em órgãos de metabolização e excreção de xenobióticos, tais como fígado e rim. Acreditamos que, além do estresse oxidativo, como vem sendo relatado na literatura, a resposta inflamatória e a ativação de vias

de morte celular detenham um papel importante na etiopatogenia do crack. Deste modo, a presente pesquisa buscará investigar se a exposição subaguda ao crack seria capaz de induzir lesões celulares no contexto inflamatório, oxidativo e proliferativo em múltiplos órgãos de ratos expostos a essa droga.

Essa dissertação foi estruturada em quatro capítulos. O **Capítulo I** será apresentado uma mini revisão bibliográfica sobre os possíveis efeitos do crack no que tange a mutagenese, estresse oxidativo, inflamação, proliferação e morte celular. O **Capitulo II** apresentamos um trabalho recentemente publicado na revista *Environmental Science and Pollution Research* sob o titulo *Genotoxicity, oxidative stress, and inflammatory response induced by Crack-Cocaine: relevance to carcinogenesis*.

Nos **Capítulos III** e **IV**, serão apresentados os resultados gerados pelo delineamento experimental executado no estudo in vivo. Parte dos resultados obtidos (*Histopathological and inflammatory response in multiple organs of rats exposed to crack)*, foi aceito para publicação na revista *International Journal of Environmental Health Research*. Por fim, no **Capítulo IV**, os resultados referentes a investigação do potencial mutagênico e a atuação da Via de Sinalização dos Receptores do Tipo Toll Like foram abrigados no segundo artigo, cujo titulo é "*Genomic instability supresses Toll Like Signaling Pathway in rat liver exposed to Crack Cocaine*", aceito para publicação na revista *In Vivo.*

2. OBJETIVOS

O objetivo da presente pesquisa foi investigar os efeitos do crack em fígado e rim de ratos Wistar machos expostos a doses subagudas de crack. Para isso foram avaliados os seguintes parâmetros:

- Análise histopatológica a partir de coloração em H.E;
- Avaliação da atividade proliferativa e pró-apoptótica por meio da Imuno-histoquímica (Ki-67, caspase-3-clivada e 8-OHdG);
- Mensuração do potencial inflamatório (TNF-α, IL-1β, IL-6) e anti-inflamatório (IL-10) pelo Ensaio ELISA;
- Mensuração da atividade da Via do Toll Like (MyD88, TRAF-6 e p-NF-κB p65) por Western Blotting (tecido hepático);
- Avaliação do potencial mutagênico pelo Teste do Micronúcleo (tecido hepático);
- Possíveis relações entre os parâmetros acima.

CAPÍTULO I

1. REVISÃO DE LITERATURA

1.1 Drogas ilícitas: Epidemiologia do Crack e Cloridrato de Cocaína

O termo “droga” é definido como qualquer substância, sintética ou natural, capaz de alterar processos bioquímicos e fisiológicos quando são introduzidas em um organismo vivo (Panus *et al.*, 2012). As drogas ilícitas são aquelas fabricadas sem o aval de um sistema regulamentador governamental, cuja comercialização é proibida por lei (Horta *et al.*, 2018).

Em geral, essas substâncias são utilizadas com o objetivo de obter um efeito psicoativo recreativo, sem qualquer orientação terapêutica. Por essa razão, há uma maior probabilidade de se estabelecer uma dependência física ou mesmo psicológica, afetando a qualidade de vida do indivíduo (Rang *et al.*, 2016). No Brasil, grande parte das drogas ilícitas, são consideradas drogas psicotrópicas, haja visto exercem um efeito sobre o sistema nervoso central. Essas são classificadas perante a atividade depressora, perturbadora ou estimuladora do sistema nervoso (Carlini *et al.*, 2001).

Acredita-se que o crack tenha sido visto pela primeira vez em solo brasileiro no início dos anos 90, onde ganhou grande popularidade no estado de São Paulo. Entre os anos de 2001 e 2005, a utilização do crack teve um aumento de duas vezes em sua prevalência, sendo sua utilização disseminada em diversos grupos socioeconômicos. Ademais, pesquisas apontam o Brasil como o maior mercado de crack do mundo (Ribeiro & Laranjeira, 2012; ABP, 2012). De fato, grandes quantidades de cloridrato de cocaína e crack são contrabandeadas na América do Sul, em direção a América do Norte e Central. O Brasil possui uma longa costa com acesso ao Oceano Atlântico, facilitando, portanto, o tráfico em direção aos continentes Africano e Europeu (UNODC, 2013).

No ano de 2012, foi realizado o II Levantamento de Nacional de Álcool e Drogas, que avaliou o consumo de drogas ilícitas pela população brasileira. Os dados revelaram que nos últimos 12 meses, cerca de 0,7% da população adulta admitiram consumir o crack. Quando foi considerado o consumo dessa droga somente uma vez na vida, foi observado a taxa de 1,3% da população estudada. Em relação aos jovens (14 a 17 anos), o consumo nos últimos 12 meses foi detectado em 0,1% dos entrevistados, sendo 0,8% quando o parâmetro foi somente uma vez na vida (LENAD, 2014). Tais resultados estão apresentados na Figura 1.

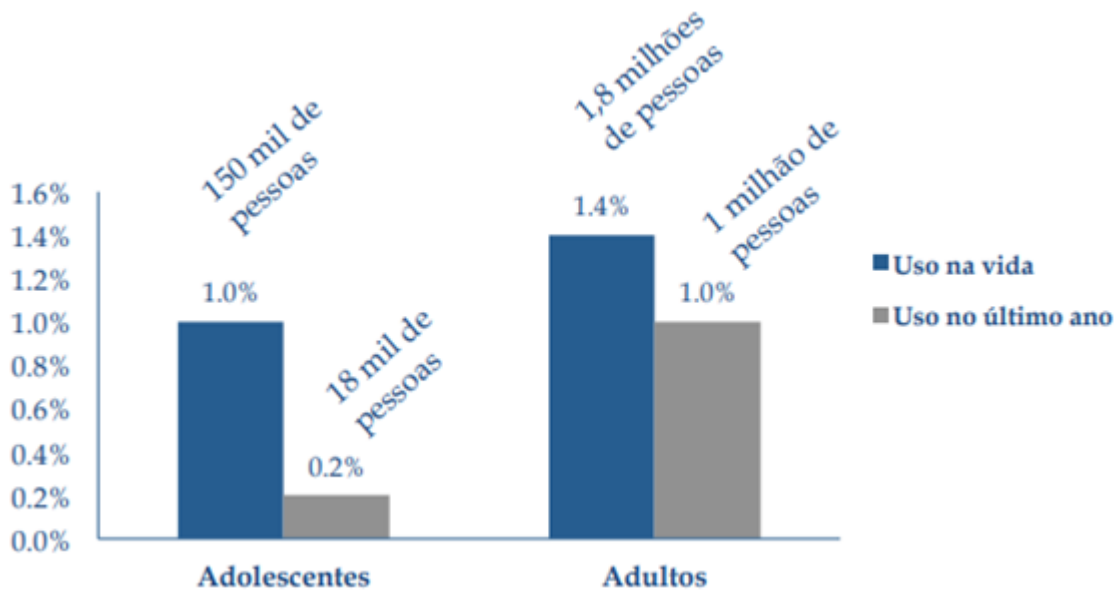

Figura 1 – Consumo de crack na vida e no último ano entre adultos e adolescentes, 2012.
Fonte: II Levantamento Nacional de Álcool e Drogas (LENAD) – 2014.

Em relação ao cloridrato de cocaína, o mesmo levantamento revelou que, o uso do entorpecente somente uma vez na vida pela população adulta foi de 3,8% da população total. No período dos últimos 12 meses, 1,7% do total a consumiam regularmente. No caso dos jovens, 2,3% dos entrevistados declararam ter usado cloridrato de cocaína pelo menos uma vez na vida; Nos últimos 12 meses, 1,6% dos jovens utilizaram essa droga regularmente (LENAD, 2014). Tais dados estão demonstrados na **Figura 2**. Em 2014, aproximadamente 6 milhões de brasileiros tiveram contato com drogas a base de cocaína pelo menos uma vez na vida (Ribeiro *et al.*, 2016).

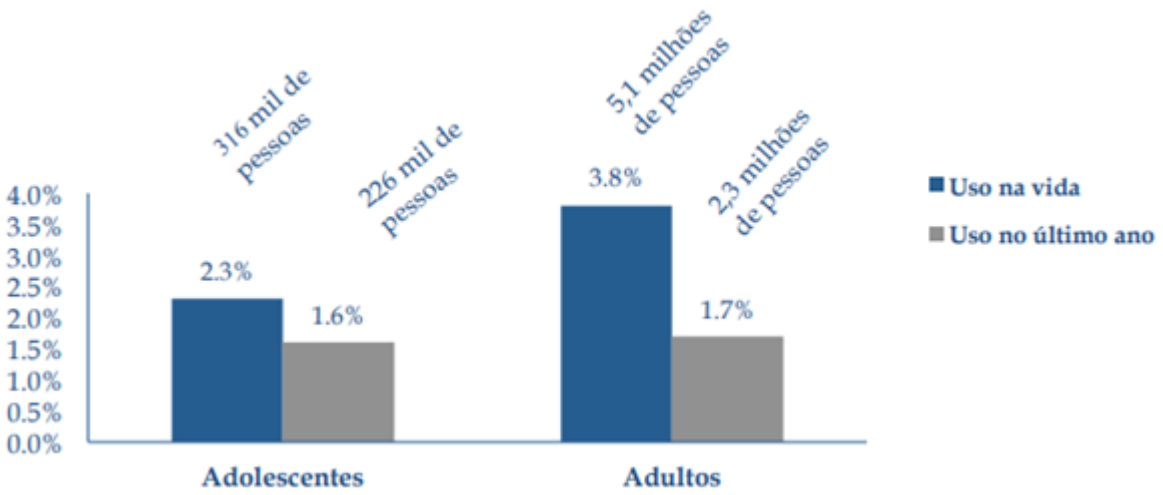

Figura 2 – Consumo do cloridrato de cocaína na vida e no último ano entre adultos e adolescentes, 2012.
Fonte: II Levantamento Nacional de Álcool e Drogas (LENAD) – 2014.

De acordo com a Receita Federal (2020), em 2019, houve um aumento de 84,08% na apreensão de cloridrato de cocaína em relação ao ano anterior (2018). No primeiro trimestre de 2020, houve um aumento de 15,3% em relação à média registrada entre janeiro e março de 2019, com um total de 14,8 toneladas de cloridrato de cocaína, sendo a maior apreensão registrada na história do nosso país em um pequeno decurso de tempo (3 meses) (**Figura 3**). Grande parte dessas apreensões (93%) ocorreram em regiões portuárias, principalmente em Santos, cidade litorânea do estado de São Paulo (Receita Federal, 2020).

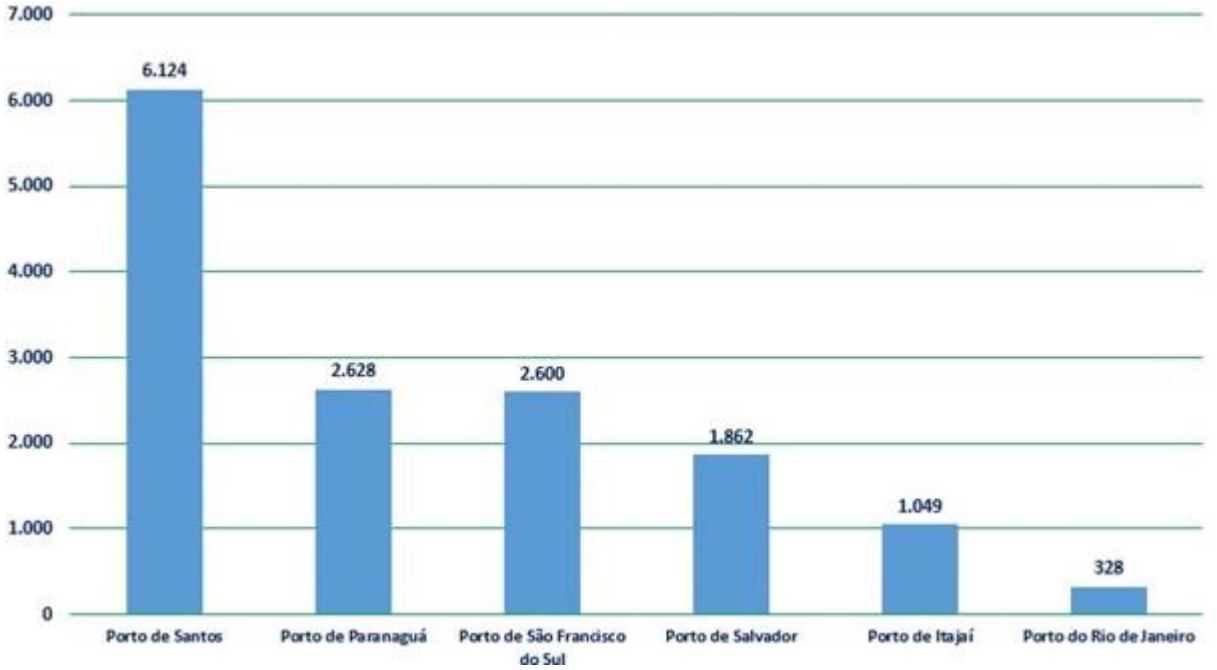

Figura 3 – Quantidade (Tonelada) de Cloridrato de cocaína apreendida no primeiro trimestre de 2020.
Fonte: Receita Federal, 2020.

Em relação ao primeiro semestre de 2020, foram apreendidas cerca de 27,8 toneladas de cloridrato de cocaína e três toneladas de crack no Brasil, representando um aumento de 5,07% em relação ao ano de 2019 (Governo do Brasil, 2020; Observatório do Crack, 2020).

1.2 *Erythroxylum coca*

Existem cerca de 250 espécies da planta coca, dentre as espécies, a *Erythroxylum coca* é a mais conhecida, sendo um arbusto de origem sul-americana (Ribeiro & Laranjeira, 2012) pertencente ao gênero *Erythroxylum* da família *Erythroxylaceae* (Barbosa *et al.*, 2014). Vale ressaltar que a ligação dos seres humanos com substâncias químicas, ocorre desde os tempos mais longínquos. O uso da cocaína, por exemplo, tem suas raízes conectadas com as grandes civilizações pré-colombianas dos Andes (Ferreira & Martini, 2001). Há registros que o cultivo dessa planta fora realizado há mais de 8.000 anos (Docimo *et al.*, 2013). Para esses povos, as plantas desse gênero (*Erythroxylum)* tinham significados místicos e religiosos, ao passo que as antigas civilizações Incas acreditavam que o ato de mascar as folhas concediam aos seus consumidores poderes mágicos (Ferreira & Martini, 2001; Barreto, 2013).

O nome “coca” é derivado do *Inca* “Cuca”, ou do *Ayamaran* “Khoka”, que significa árvore (Muakad, 2011). Apesar de ser ilegal em diversos países, essa planta ainda é cultivada legalmente em volumes controlados na Bolívia, Colômbia e Peru, onde somente as folhas da coca são permitidas, sendo o processo de refino considerado um ato totalmente ilegal (Ribeiro & Laranjeira, 2012; Passagli, 2013). Questões climáticas (temperaturas entre 15 a 20ºC, regiões entre 2600 a 2800 metros acima do nível do mar e a acidez do solo) e culturais (hábito de mascar essas folhas) justificam o plantio nesses países (Passagli, 2013; Muakad, 2011).

O cultivo da coca ocorre, em um primeiro momento, em sementeiras e somente depois de alguns meses ocorre a transferência para campos de plantação direta. Esse arbusto alcança cerca de 3 metros de altura, possuí folhas em formatos ovais com tamanhos que variam de 6 cm de comprimento por 3 cm de largura (**Figura 4**), contendo várias substâncias aromáticas e uma resina perfumada (Muakad, 2011).

Figura 4 – Folhas da *Erythroxylum coca*
Fonte: Adaptado de Docimo *et al.*, 2013.

Dentre as espécies inclusas nessa família, a *Erythroxylum coca* ganha um certo destaque, devido a sua grande utilização para obtenção de cocaína, visto que dentre as outras espécies, essa possui uma maior concentração desse alcaloide (Mendonça *et al.*, 1998),

representando aproximadamente de 20 a 90% do principal alcaloide encontrado nessa planta (Oliveira & Dinis-Oliveira, 2018).

Os alcaloides são compostos nitrogenados de origem vegetal que possuem um potencial farmacológico bem estabelecido. Existem cerca de 12.000 tipos de alcaloides presentes em diversas plantas espalhadas pela natureza (Yazaki *et al.*, 2008). Um exemplo de alcaloide é a cocaína, que nas plantas atua como uma substância protetora, impedindo ataques de herbívoros, patógenos e toxinas em geral (Torre *et al.*, 2013). Apesar de possuírem uma atividade biológica importante no reino vegetal, os alcaloides apresentam atividade citotóxica para diversos mamíferos e outros vertebrados. Cumpre notar que, existem alguns fatores importantes no universo da toxicologia quando se remete à intoxicação, tais como, a dose e a susceptibilidade do organismo alvo (Matsuura & Fett-Neto, 2015).

Em 1855, Freidrich Gaedcke foi o primeiro a produzir um extrato das folhas da coca, sendo nomeado de *erythroxylene.* Alguns anos depois, em 1859, o químico alemão Albert Niemann caracterizou e isolou um alcaloide especifico das folhas, em decorrência de uma maior concentração em relação aos demais. Niemann nomeou esse determinado composto como *cocaína* (Ponce, 2015). Por volta de 1884, a cocaína começou a ser utilizada na medicina ocidental, em especial pelos médicos austríacos Karl Koller e Sigmund Freud, sendo largamente utilizada e popularizada como um potente estimulante e anestésico local, recomendada no tratamento para exaustão, histeria, algumas infecções e até mesmo para retirar a dependência de outra substância. Desse modo, quando descoberta, a cocaína fora muito utilizada na medicina devido suas propriedades anestésicas, e dessa forma, começaram a prescrevê-la, em particular, para doenças de difícil tratamento (Ponce, 2015; Ferreira & Martini, 2001; Passagli, 2013).

Por volta de 1885, a cocaína passou a ser incorporada a bebidas alcoólicas, bebidas açucaradas, cigarros e alguns medicamentos. No ano de 1863, um químico chamado Angelo Mariano produziu um vinho a partir do extrato de cocaína, o Vin Mariani. A partir disso, a

cocaína tornou-se um dos ingredientes mais populares para produção de bebidas em geral. Em 1886, John Styth Pemberton inventa a fórmula de uma bebida açucarada, a Coca-Cola. A receita de Pemberton passa a ser um dos principais concorrentes do Vin Mariani, pois prometia as virtudes da cocaína sem os vícios do álcool. Entretanto, episódios de toxicidade e óbitos começaram a ser relatados em diversas revistas médicas por volta de 1920, devido a popularização do consumo de produtos à base de cocaína (Ferreira & Martini, 2001; Goldstein *et al.*, 2009; Ribeiro & Laranjeira, 2012).

Atualmente, a cocaína possui alguns padrões de uso conforme sua forma de apresentação. De acordo com Ribeiro e Laranjeira (2012), o processo de refinamento das folhas da *Erythroxylum coca* pode dar origem a algumas das suas formas de apresentação (**Figura 5**). Por meio da maceração das folhas e adição de alguns compostos químicos, obter-se-á dois produtos: a pasta de coca ou a *merla*. A pasta de coca corresponde à droga em sua forma de base; sendo o produto original, após os primeiros tratamentos com as folhas de coca (Andrade Filho *et al.*, 2013; Lopez-Hill *et al.*, 2011).

A *merla* (mela, mel ou melado), encontra-se também sob a forma de base, de coloração amarelada e inodora, é obtida a partir de um refino mais grosseiro em relação a pasta de coca, em virtude da contaminação com diversos produtos químicos utilizados na extração da cocaína (CEBRID, 2012). A partir da pasta de coca, obter-se-á o cloridrato de cocaína, que possui um aspecto de sal de coloração branca ou acinzentado, coloquialmente denominada de “pó”, “farinha”, “neve” e/ou “branquinha”. O cloridrato de cocaína corresponde à droga na sua forma de sal. Tanto a pasta de coca, quanto ao cloridrato de cocaína, dão origem ao crack (Passagli, 2013; Oliveira, 2017). A **Figura 5** demonstra o processo de refinamento das folhas de coca para obtenção de drogas ilícitas. Basicamente, todo o processo reside na maceração e adição de algumas substâncias químicas, quanto mais o produto for refinado, maior será o grau de pureza de cocaína nas novas formulações (Oliveira & Nappo, 2008).

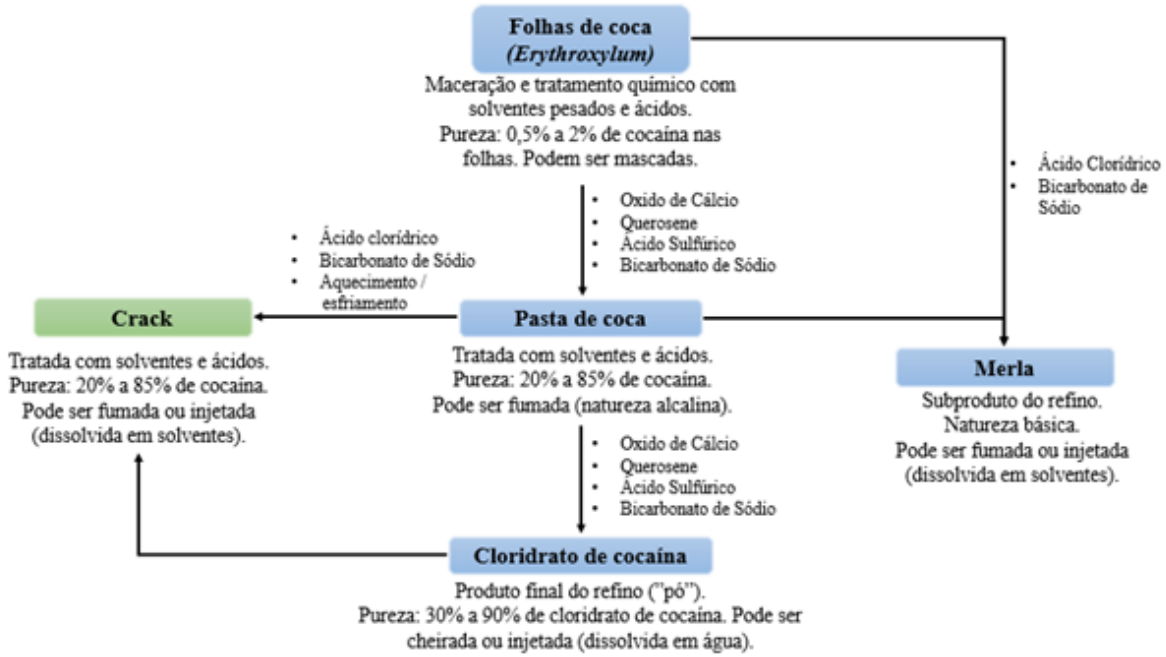

Figura 5 – Processo do refinamento da cocaína.
Fonte: Adaptado de Corrêa *et al.*, 2014 e Oliveira, 2017.

1.3 Crack

O crack é a cocaína em sua forma mais deletéria, devido a presença de resquícios de outros agentes químicos (adulterantes), que são utilizados durante o processo de refinamento (Passagli, 2013; Riezzo *et al.*, 2012). Essa droga apresenta-se, quimicamente, sob a forma de base. A denominação "*crack"* é utilizada devido a expressão onomatopaica que remete ao som de crepitação gerado durante a pirólise, quando a droga é consumida por meio do fumo (Oliveira & Dinis-Oliveira, 2018). O crack é considerado uma das drogas ilícitas mais utilizadas ao redor do mundo, e possui um aspecto de cristais irregulares em forma de "pedras", de coloração marrom, e/ou bege amarelado (**Figura 6**) (Andrade Filho *et al.*, 2013; Oliveira, 2017).

Figura 6 – Pedras de crack.
Fonte:< http://cocaine.org/hardstuf.html>.

O crack surgiu em meados da década de 80, sendo identificado pela primeira vez nos Estados Unidos da América (EUA) (Bastos & Bertoni, 2014). Em 1996, o perfil do consumidor dessa droga fora relatado, sendo caracterizado, em grande parte das vezes, como homens jovens de baixo nível de escolaridade e sem vínculo empregatício formal (Oliveira & Nappo, 2008).

Grande parte dos usuários, utilizam essa droga por um tempo prolongado, levando o indivíduo a um esgotamento físico, psíquico e financeiro. A fissura por essa droga é responsável pela perda de interesse por atividades essenciais, como o sono, a alimentação e senso de responsabilidade. Especialmente, as questões financeiras podem levar o usuário a participar de atividades ilícitas, como o tráfico, roubos ou furtos (Oliveira & Nappo, 2008).

1.3.1 Toxicocinética e Toxicodinâmica do Crack

O surgimento de sinais e sintomas, que caracterizam a quebra da homeostasia do organismo frente a um xenobiótico, é conhecido como intoxicação. A intoxicação pode ser dividida em quatro processos distintos: exposição, toxicocinética, toxicodinâmica e clínica (Passagli, 2013).

A fase de exposição corresponde ao momento de contato do xenobiótico com o organismo, levando em consideração o período de tempo de exposição. A fase da toxicocinética

consiste no movimento do xenobiótico dentro do organismo, tal processo inclui a absorção, distribuição, biotransformação, excreção e/ou armazenamento. A toxicodinâmica representa a ação do xenobiótico no organismo, fase onde o xenobiótico ou metabólito ativo (produto da biotransformação) interage com o tecido alvo, podendo levar a alterações morfofuncionais. Por fim, a fase clínica, representa efeitos adversos produzidos pelo xenobiótico, ou seja, os sinais e sintomas que determinam a intoxicação propriamente dita (Sprada, 2013; Passagli, 2013).

O principal componente químico do crack (cocaína) possui uma boa absorção por grande parte das vias de administração, sendo o tempo uso-efeito totalmente dependente da maneira como a droga é utilizada (Castro *et al.,* 2015). O principal meio de consumo do crack ocorre pelo fumo (via pulmonar) com o auxílio de cachimbos. Tais objetos, em muitas das vezes, são de fabricação própria, confeccionados a partir de copos, tubos e garrafas plásticas, lâmpadas, canos de encanamento e latas de alumínio (Oliveira & Nappo, 2008).

Quando essa droga é utilizada por meio do fumo, a cocaína sofre uma rápida absorção pelos alvéolos pulmonares devido a alto fluxo sanguíneo presente nas extensas redes de capilares na região (Chasin *et al.,* 2014). Além disso, esse composto apresenta um baixo ponto de fusão e, durante o fumo ocorre facilmente uma decomposição térmica (pirólise), cujo resultado é a conversão da maior parte em metilecgonidina. Essa, pode ocorrer entre 50 a 80% em temperaturas superiores a 250ºC (Paul *et al.,* 2005).

Entretanto, a via pulmonar pode ser comparada com a via intravenosa devido ao rápido surgimento dos efeitos, podendo ser imediato e/ou atingindo um pico em apenas 5 minutos, com duração de aproximadamente 30 minutos, após a exposição (Waninger *et al.*, 2007; Lankenau *et al.,* 2004; Harris *et al.*, 2019). Após a absorção, a cocaína é distribuída por todo o organismo via corrente sanguínea, onde cerca de 70% do que fora absorvido circula de maneira livre, sem se ligar a proteínas plasmáticas. O restante liga-se a algumas proteínas plasmáticas,

como a α-1-glicoproteína ácida e albumina, com uma alta afinidade pelo tecido cerebral e hepático (De Castro *et al.*, 2015).

A preferência pelo tecido cerebral pode ser explicada pelas características lipofílicas da cocaína, onde a mesma consegue atravessar facilmente a barreira hematoencefálica. Entretanto, o acúmulo dessa substância no fígado é justificado por duas hipóteses, a primeira é o principal órgão de detoxificação; a segunda hipótese é que os hepatócitos possuem receptores com alta afinidade pela cocaína, porém os efeitos biológicos ainda não estão totalmente esclarecidos (Chasin *et al.*, 2014; De Castro *et al.*, 2015). Nesse contexto, pesquisas nesse campo de estudo são necessárias para se entender os reais efeitos exercidos por esse entorpecente em órgãos metabolizadores como o fígado.

O início da biotransformação da cocaína ocorre, em primeira instancia, pela ação das colinesterases plasmáticas e se encerra no fígado, onde é degradada a outros metabólitos (**Figura 7**), como a benzoilecgonina, éster-metilecgonina e norcocaína (De Castro *et al.*, 2015).

A benzoilecgonina surge por hidrólise espontânea ou devido a ação enzimática das carboxilesterases, correspondendo a 45% dos metabólitos da cocaína. A éster-metilecgonina representa 40% dos subprodutos, sendo sintetizada por meio da hidrólise da cocaína pelas enzimas butirilcolinesterases (pseudocolinesterases). Em um menor percentual, temos a norcocaína que é originada pelo processo de *N*-desmetilação oxidativa, catalisada pelas enzimas presentes na família citocromo P450 (CYP450) (Ponce, 2015; Oliveira & Dinis-Oliveira, 2018). A principal via de excreção ocorre através da via urinária (renal), seja na forma da cocaína propriamente dita, ou sob a forma de seus metabólitos (Goldstein *et al.*, 2009).

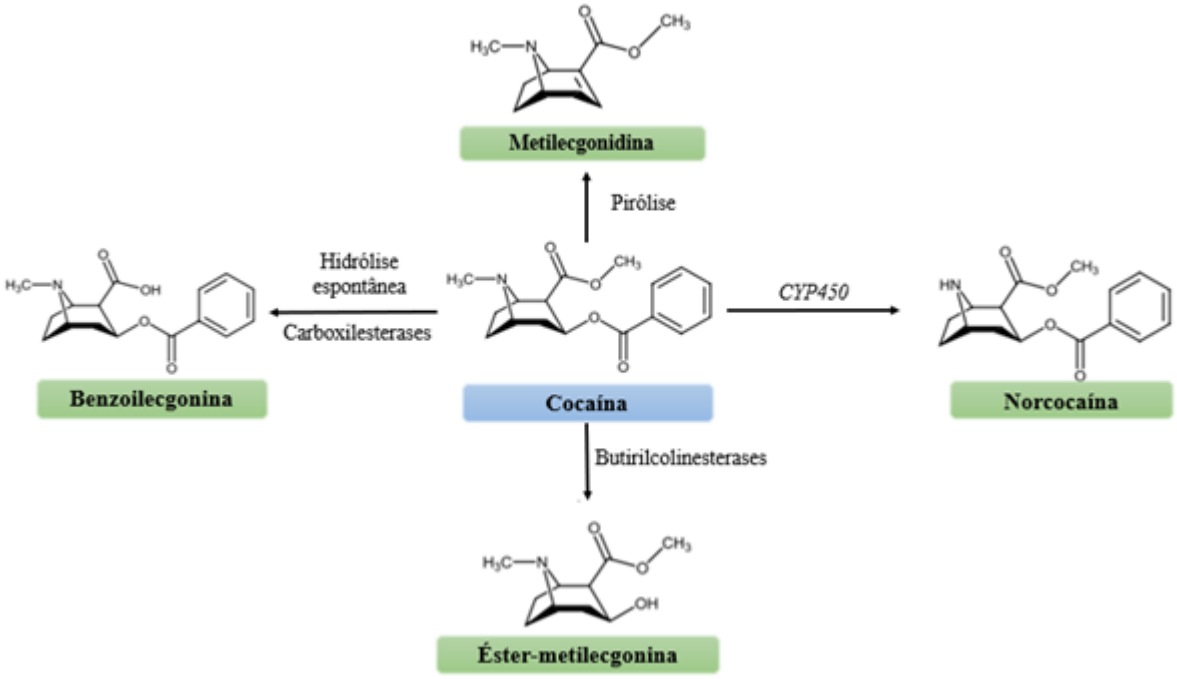

Figura 7 – Principais metabólitos da cocaína.
Fonte: Adaptado de Oliveira & Dinis-Oliveira, 2018.

No sistema nervoso central, o principal constituinte químico do crack (cocaína) bloqueia a recaptação de alguns neurotransmissores, em especial das monoaminas, como a dopamina, serotonina e noradrenalina, gerando um acúmulo dessas substâncias na fenda sináptica, cujo resultado é estimulação constante dos neurônios pós-sinápticos, que resultam nas sensações de euforia. Contudo, o uso contínuo dessa droga poderá gerar o esgotamento desses neurotransmissores, levando o indivíduo a quadros de distúrbios psiquiátricos, como por exemplo, a depressão e/ou ansiedade (Dinis-Oliveira *et al.*, 2012; Oliveira & Dinis-Oliveira, 2018).

O consumo do crack por meio do fumo pode levar, em pouco tempo, a dependência do usuário, em virtude do princípio ativo atingir altas concentrações plasmáticas em poucos instantes, no sistema nervoso central, gerando a liberação e retenção de neurotransmissores na fenda sináptica (Passagli, 2013). Deste modo, acredita-se que o usuário possa sofrer alterações morfológicas no cérebro (neuroplasticidade), que envolvem a criação de novos receptores para

atender a alta demanda de neurotransmissores retidos na fenda sináptica favorecendo, assim, o desenvolvimento da dependência química (Ribeiro & Laranjeira, 2012; Ferreira *et al.,* 2017; Rosário *et al.,* 2019).

A adaptação dos receptores cerebrais devido à presença frequente dos neurotransmissores, ainda pode gerar uma diminuição da potência da droga. Tal fato é justificado pois, frente a uma nova exposição, a quantidade de neurotransmissores liberados não são suficientes para um estímulo eficiente dos neurônios pós-sinápticos, devido à uma maior expressão de receptores, o que faz o indivíduo buscar um consumo de uma quantidade cada vez maior para que o mesmo efeito bioquímico anterior seja almejado (Ferreira *et al.*, 2017).

No sistema nervoso central, um estudo recente com ratos, verificou os efeitos de diferentes doses de crack. Neste estudo, os ratos foram expostos por via intraperitoneal (i.p) as doses de 18, 25 e 36 mg/kg de crack 1 vez ao dia por 5 dias consecutivos. Os dados revelaram que a menor dose (18 mg) gerou um efeito panicolítico (ausência de medo/pânico), consequentemente, uma menor imunoexpressão de DeltaFosB (marcador de atividade neural). Efeitos opostos foram ainda observados na dose de 25 e 36 mg, visto que o crack promoveu um efeito panicogênico (medo/pânico excessivo), com um aumento da expressão de DeltaFosB em certas regiões encefálicas (Rosário *et al*., 2021).

Além do sistema nervoso central, distúrbios respiratórios foram relatados pelo consumo do crack por meio do fumo, podendo gerar quadros de sinusite, dispneia, rinorréia, granulomas pulmonares (Passagli, 2013). Efeitos no sistema cardiovascular também foram relatados anteriormente na literatura, e correlacionados a dois fatores: o bloqueio da recaptação de noradrenalina e potencial anestésico da cocaína com o bloqueio de canais de sódio, levando ao aumento da frequência cardíaca e vasoconstrição na musculatura cardíaca. Acredita-se ainda, que outros efeitos deletérios do crack estariam envolvidos, sendo o estresse oxidativo o mais

aceito atualmente (De Castro *et al.*, 2015; Goldstein *et al.*, 2009; Oliveira & Dinis-Oliveira, 2018).

De fato, pesquisadores acreditam que o crack possa ter um efeito deletério mais potente por ser considerado um produto mais "sujo" em relação ao cloridrato de cocaína (Oliveira & Dinis-Oliveira, 2018). Independentemente da forma de apresentação dessas drogas, o fígado e os rins são os principais órgãos de metabolização e excreção de xenobióticos, sendo assim, são órgãos considerados críticos quando se investigam agentes quimicos, especialmente àqueles considerados suspeitos (Vitcheva, 2012; Sales & Foresto, 2020).

1.4 Estresse Oxidativo

As espécies reativas de oxigênio (ERO's) são moléculas remanescentes do metabolismo celular, de meia vida curta e extremamente reativas (Cloux *et al*., 2019), que apresentam atividade biológica importante em eucariotos. Algumas dessas espécies reativas, atuam em processos de sinalização celular, alteração dos padrões de expressão gênica, fagocitose, e síntese de determinadas substâncias (Barreiros *et al.*, 2006), além da regulação do ciclo celular, apoptose, autofagia e senescência celular (Abdalla, D. *et al.*, 2014).

A principal molécula responsável pela origem das ERO's é o oxigênio (O_2) em seu estado fundamental (molécula que respiramos), que é metabolizado de duas maneiras: aproximadamente 90% das vezes o O_2 é utilizado pelas mitocôndrias na cadeia respiratória mitocondrial (transporte de elétrons) durante a fosforilação oxidativa para a obtenção de energia (ATP – adenosinatrifosfato); e cerca de 10% são usados em reações químicas de oxidação enzimática (Schneider & Oliveira, 2004; Hirata *et al.*, 2004).

A formação das ERO's pode estar associada aos processos metabólicos (endógeno) e/ou devido a processos exógenos, como por exemplo: exposição à radiação ultravioleta, poluição e ao uso de drogas. Assim, a produção de ERO's podem ocorrer naturalmente ou induzida por

fatores externos, sendo O_2 responsável pela geração de grande parte dessas moléculas reativas. Dentre elas, destacam-se o íon superóxido (O_2•-), o radical hidroxila (HO•) e o peróxido de hidrogênio (H_2O_2) capazes de promover oxidação da bicamada lipídica das membranas celulares, danos a proteínas e ao DNA, provocando diversas alterações na função celular e, portanto, tecidual (Angeli, 2011; Vasconcelos *et al.,* 2014).

Conforme anteriormente relatado, as ERO's em até certo ponto possuem um papel estabelecido no organismo, isso porque o organismo possui mecanismos de defesa antioxidante para limitar os níveis intracelulares das mesmas, impedindo, portanto, que essas moléculas causem danos teciduais significativos (Bianchi & Antunes, 1999). Porém, quando há um desequilíbrio entre a concentração intracelular de moléculas antioxidantes e pró-oxidantes, inicia-se um processo denominado de estresse oxidativo (Vasconcelos *et al.,* 2014). Deste modo, o termo estresse oxidativo é empregado em circunstâncias nas quais as altas concentrações de ERO's se sobrepujam ante ao processo antioxidante, causando danos oxidativos a estruturas celulares (Schneider & Oliveira 2004).

Danos oxidativos no DNA, levam à produção de 8-hidroxi-2'-desoxiguanosina (8-OHdG), devido a oxidação da base nitrogenada guanina. De primeiro momento, ocorre a adição do HO• no carbono de posição 8, dando origem a um aduto de DNA, o 8-Hidroxiguanina (8-OHGua). Após esse processo, essa molécula acaba sofrendo processos de reparo, dando origem ao 8-OHdG, considerado um importante biomarcador de dano ao DNA (Valavanidis *et al.,* 2009; Ribeiro *et al.,* 2007; Kasai, 2016). Fatores endógenos, como a idade; e fatores exógenos, como dieta, exposição a drogas e poluentes, podem variar os níveis de danos oxidativos do DNA. Entretanto, níveis aumentados de 8-OHdG têm sido encontrados em diferentes doenças crônico-degenerativas, como por exemplo, as neoplasias (Cunha *et al.,* 2017).

Estudos recentes vêm demonstrando que o cloridrato de cocaína seria capaz de gerar estresse oxidativo em diversos órgãos, por meio de uma superprodução de espécies reativas de

oxigênio, cujo resultado é a peroxidação da bicamada fosfolipídica (membrana celular), oxidação de proteínas e danos oxidativos ao DNA. Além disso, alguns autores têm relevado que o crack promove a deficiência de diversas enzimas atuantes no sistema antioxidante, tanto em âmbito celular como sistêmico (Vasconcelos *et al.*, 2014; Yujra *et al.*, 2015). Certamente, os tipos de lesões, bem como a frequência com que elas ocorrem devido ao consumo dessa droga, variam de acordo com a dose e susceptibilidade individual, haja visto diferentes tecidos podem apresentar respostas adaptativas distintas (Yujra *et al.*, 2015). Diante dessas consequências, o consumo dessa droga poderia gerar diversas lesões teciduais, visto que o crack é repleto de adulterantes, além da cocaína.

1.5 Resposta Inflamatória

O sistema imunológico é composto por uma gama de tipos celulares e moléculas, que têm por função garantir a proteção do organismo contra a presença de patógenos. O mesmo está organizado em resposta imune inata e adaptativa (Cruvinel *et al*., 2010). A resposta inata é responsável por uma reação inflamatória aguda que é acompanhada de vasodilatação sistêmica, extravasamento vascular seguida de emigração leucocitária. Em contrapartida, a resposta imune adaptativa é caracterizada pela atividade dos linfócitos T e B, os quais reconhecem determinados antígenos e fornecem respostas rápidas e específicas devido a memória imunológica (Clark & Kupper, 2005).

Ambas as respostas imunológicas (inata e adaptativa) são dependentes de células do sistema imune (monócitos/macrófagos, linfócitos, entre outras) e não imunes (fibroblastos e células endoteliais), para uma proteção efetiva do organismo. Tais funções são essenciais na proteção contra o desenvolvimento de infecções e tumores (Cordova Martinez & Alvarez-Mon, 1999; Cruvinel *et al*., 2010).

A inflamação é um mecanismo essencial que garante a proteção do organismo frente a agentes biológicos, físicos e químicos, como por exemplo entorpecentes em geral. Sabe-se que a inflamação, também atua no processo de regeneração tecidual. Uma vez que a integridade do tecido é restaurada, o processo inflamatório cessa, entretanto, caso haja alguma desregulação, a inflamação permanece, transformando-se em um processo crônico (Oishi & Manabe, 2018).

Algumas citocinas são classificadas como pró- e outras como anti-inflamatórias. Dentre as que induzem a atividade inflamatória, destaca-se o fator de necrose tumoral alfa (TNF-α) e as interleucinas 1 beta e 6 (IL-1β e IL-6), enquanto a interleucina 10 (IL-10) apresenta atividade anti-inflamatória (Souza *et al*., 2010). Drogas de abuso a base de cocaína, podem afetar o sistema imunológico alterando os níveis das citocinas participantes do processo inflamatório (Yamada & Nabeshima, 2004).

Até o presente, são escassos os estudos investigando a atividade inflamatória em decorrência da exposição ao crack. Narvaez e colaboradores (2013), verificaram uma elevação plasmática da IL1β, TNF-α e IL-10 em estudo com humanos, após exposição do crack e cloridrato de cocaína. Em outro estudo, com humanos, Gan e colaboradores (1998) encontrou níveis plasmáticos da IL-10 diminuídos, após exposição ao cloridrato de cocaína. Por outro lado, Irwin e colaboradores (2007) verificaram uma diminuição na concentração plasmática da IL-6 em indivíduos dependentes do cloridrato de cocaína após dois dias de exposição. Entretanto, não é conhecido até o presente, o comportamento das citocinas participantes da inflamação diante da exposição ao crack em outros tecidos e/ou órgãos, tais como fígado e rim.

1.5.1 Via dos Receptores do Tipo Toll Like

Os receptores do tipo toll like (TRL) são uma classe de receptores transmembranares descritos originalmente em insetos do gênero *Drosaphila*. Incialmente, verificaram sua atuação

no processo de embriogênese e, posteriormente, a atividade contra infeções fúngicas (Takeda *et. al.*, 2003; Kawai & Akira, 2004).

Atualmente, é reconhecido pela literatura a existência desses receptores em mamíferos. Em seres humanos, existem cerca de 10 diferentes tipos de TLRs (1 ao 10) enquanto um total de 11 receptores foram descritos em murinos (Hopkins & Sriskandan, 2005). Os TRLs são uma classe importante de receptores que atuam na resposta imunológica inata, responsáveis por identificar padrões moleculares associados a patógenos (PAMPs) e padrões moleculares associados a danos celulares (DAMPs) (Kawasaki & Kawai, 2014).

Os PAMPs são moléculas constitutivas e conservadas no metabolismo de microrganismos, como por exemplo, os lipopolissacarídeos (LPS) presentes na membrana de bactérias gram-negativas. Assim, as PAMPs indicam a presença de invasores microbianos no organismo hospedeiro. Os DAMPs, contudo, atuam pro meio do estresse ou lesões teciduais. A presença dessas moléculas em microambientes anômalos caracterizam sinais de alerta, cujo resultado é a deflagração da resposta inflamatória (Medzhitov, 2001; Roh & Sohn, 2018).

Para esses receptores, de fato, induzirem uma resposta inflamatória, os TRLs precisam ser ativados, a partir de uma cascata de eventos moleculares que irão culminar com a ativação do fator de transcrição nuclear *kappa* B (NF-κB), capaz de ativar genes relacionados a citocinas pró-inflamatórias (Barton & Medzhitov, 2003). Os TRLs ainda podem ser classificados em dois grupos, de acordo com a proteína inicial responsável pela transdução do sinal. Deste modo, tem-se os receptores dependentes do fator de diferenciação mieloide 88 (MyD88) e os não dependentes da via MyD88 (Ferraz *et al.*, 2011).

Na via dependente do MyD88, essa proteína é recrutada e se associa a molécula adaptadora associada a receptores do tipo toll like (TIRAP). Esse complexo proteico será responsável pelo recrutamento e ativação (fosforilação) das proteínas quinases associadas ao receptor de interleucina do tipo 1 e 4 (IRAK1 e IRAK4) (Ferraz *et al.*, 2011; Pisani *et al.*, 2017).

Na via independente do MyD88, os receptores recrutam a molécula TRIF (Adaptador indutor de interferon-β) que irá induzir a ativação da proteína de interação do receptor de quinases (RIP 1) (Meylan *et al.,* 2004). A partir desse ponto, as proteínas IRAK (1 e 4) e RIP 1, terão por função recrutar e ativar o receptor associado ao fator de necrose tumoral do tipo 6 (TRAF-6) (Akira, 2003). O TRAF-6 irá ativar o fator de crescimento β associado à quinase 1 (TAK1) que irá se associar a proteína ligante de TAK do tipo 1 e 2 (TAB1 e TAB2). A interação desses complexos irá promover a ativação do complexo de quinases de IκB (IKK) (Ferraz *et al.,* 2011; Pisani *et al.,* 2017).

O NF-κB é uma proteína formada por duas subunidades: NFκBp65 e NFκBp50. As proteínas inibitórias de IκB estão ligadas ao NF-κB, tornando-o inativo. Com a ativação do complexo IKK, as proteínas inibitórias de IκB são degradas e ocorre a liberação do NF-κB (Karin, 1999), que irá migrar para o núcleo celular e promover a ativação de genes que expressam as citocinas pró-inflamatórias, tais como: TNF-α, IL-1β e IL-6 (Blanco & Condino Neto, 2003; Glezer *et al.,* 2000; Moresco *et al.,* 2011; Pisani *et al.,* 2017). A **Figura 8**, apresenta um esquema das vias dos receptores do tipo toll like dependente e independentes de Myd88.

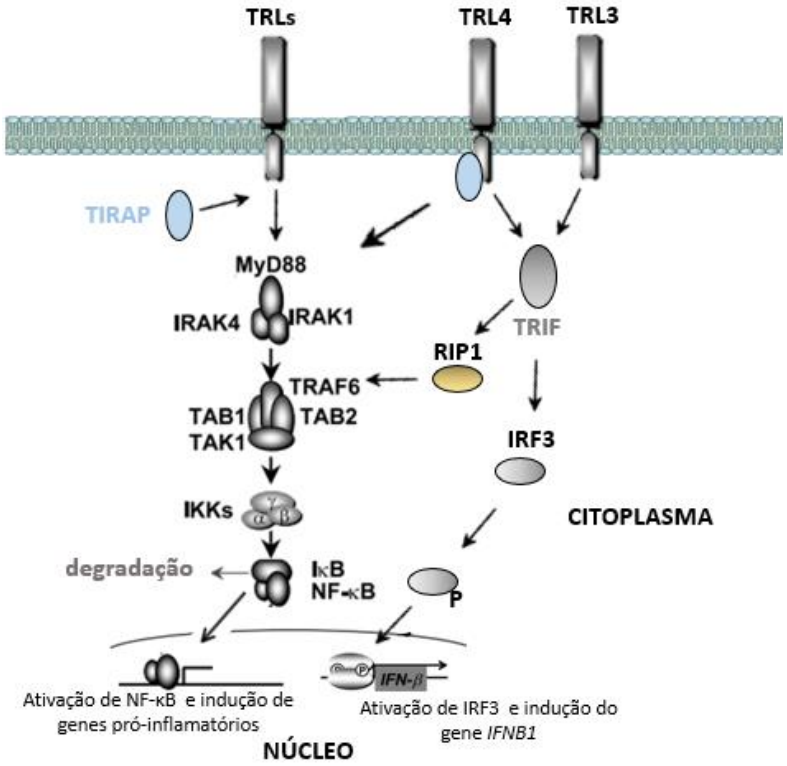

Figura 8 – Via de Sinalização dos Receptores Tipo Toll Like – Via Dependente e Independente de Myd88.
Fonte: Adaptado de Akira & Sato, 2003.

1.6 Possíveis mecanismos da hepatotoxicidade e nefrotoxicidade do Crack (cocaína)

O fígado é considerado um dos maiores órgãos do corpo humano, com funções que vão desde a metabolização, distribuição de substâncias, até a desintoxicação e síntese de proteínas, funções consideradas vitais ao organismo. Este órgão possui uma maior concentração de enzimas que atuam no metabolismo de diversas substâncias, como o complexo enzimático da CYP450, que atuam na função de desintoxicação. O complexo de enzimas CYP450, além de estar presente no tecido hepático, também pode ser encontrado em menores concentrações em outros sítios como nos rins, sangue e pulmões por exemplo (Mcdonnell & Dang, 2013; Dixon *et al.*, 2014).

Em relação a função metabolizadora do fígado, a mesma pode ser dividida em duas fases distintas, porém relacionadas. A fase I corresponde a etapa de adição ou exposição de um grupo funcional (-OH,-NH_2,-SH,-COOH) pré-existente ao xenobiótico, por meio de reações de oxidação, redução e/ou hidrólise. Esse processo tem por função a inativação ou ativação dessa substância (Huber *et al*., 2008). A fase II consiste na etapa da conjugação dos metabolitos remanescentes a outras moléculas menos tóxicas, por meio de processos como a metilação e a acetilação de xenobióticos. Deste modo, ambas as fases buscam transformá-los em compostos mais solúveis (polares) e menos reativos, a fim de facilitar a excreção e, assim, mitigar a probabilidade de agravos ao organismo. Entretanto, alguns xenobióticos podem dar origem a metabólitos nocivos à saúde, causando danos a componentes celulares e a formação de adutos de DNA (Liska *et al.*, 2006; Gonçalves *et al.*, 2014).

Após os xenobióticos sofrerem metabolização, a sua excreção ocorre através de algumas vias, como via gastrointestinal, pulmonar, dérmica, biliar e renal. A excreção de algumas drogas ocorre principalmente pela via renal. Além de exercer uma função excretora, os rins atuam, portanto, na regulação homeostática de eletrólitos, manutenção da osmolaridade do plasma e síntese de alguns hormônios (Dixon *et al.*, 2014). Deste modo, fígado e os rins são os principais órgãos de metabolização e excreção de xenobióticos (Vitcheva, 2012; Sales & Foresto, 2020).

No tecido hepático, o suposto efeito hepatotóxico da cocaína estaria associado ao estresse oxidativo, pois além da geração de ERO's durante processo de síntese de ATP, outra fonte seria responsável também pela síntese de espécies reativas, que neste caso em específico, seria o metabolismo oxidativo de xenobióticos. Em outras palavras, cabe ressaltar que o metabolismo da cocaína acaba produzindo metabólitos citotóxicos e ERO's na tentativa de excretá-la (Vitcheva, 2012).

Como dito anteriormente, quando a cocaína é absorvida pelo organismo, inicia-se o processo de metabolização. No fígado, grande parte da cocaína será metabolizada a diferentes

metabólitos (**Figura 7**), dentre eles, a norcocaína, metabólito oriundo da oxidação pelo complexo CYP450. Entretanto, esse metabólito parece dar origem aos derivados da norcocaína (**Figura 9**), envolvidos, de fato, com estresse oxidativo (Vitcheva, 2012).

O primeiro metabólito secundário da norcocaína surge por meio da hidroxilação da mesma, dando origem a *N*-hidroxinorcocaína, reação catalisada pela CYP450 dependente de flavina-adenina dinucleotídeo monooxigenases (FADM). Esse metabólito secundário (*N*-hidroxinorcocaína) induz ciclo *redox*, assim a *N*-hidroxinorcocaína é oxidada dando origem ao nitróxido de norcocaína. Quando a nitróxido de norcocaína é reduzida, retorna a *N*-hidroxinorcocaína, tal processo é dependente de CYP450, FADM e citocromo P_{450} redutase (CRP). Durante esse ciclo *redox* vicioso, ocorre a produção de ERO's, em especial, o O_2•- e o H_2O_2 (**Figura 9**). Além disso, o nitróxido de norcocaína sofre um processo de oxidação, formando a norcocaína nitrosônio. Tal composto parece estar relacionado com a depleção dos níveis intracelulares de glutationa reduzida (GSH), principal molécula do sistema de defesa antioxidante, assim, favorecendo um desiquilíbrio entre agentes antioxidantes e pró-oxidantes, contribuindo com o estresse oxidativo (Schneider & Oliveira, 2004; Kovacic, 2005; Sierra & Torres, 2005; Barbosa *et al.*, 2010; Valente *et al.*, 2012).

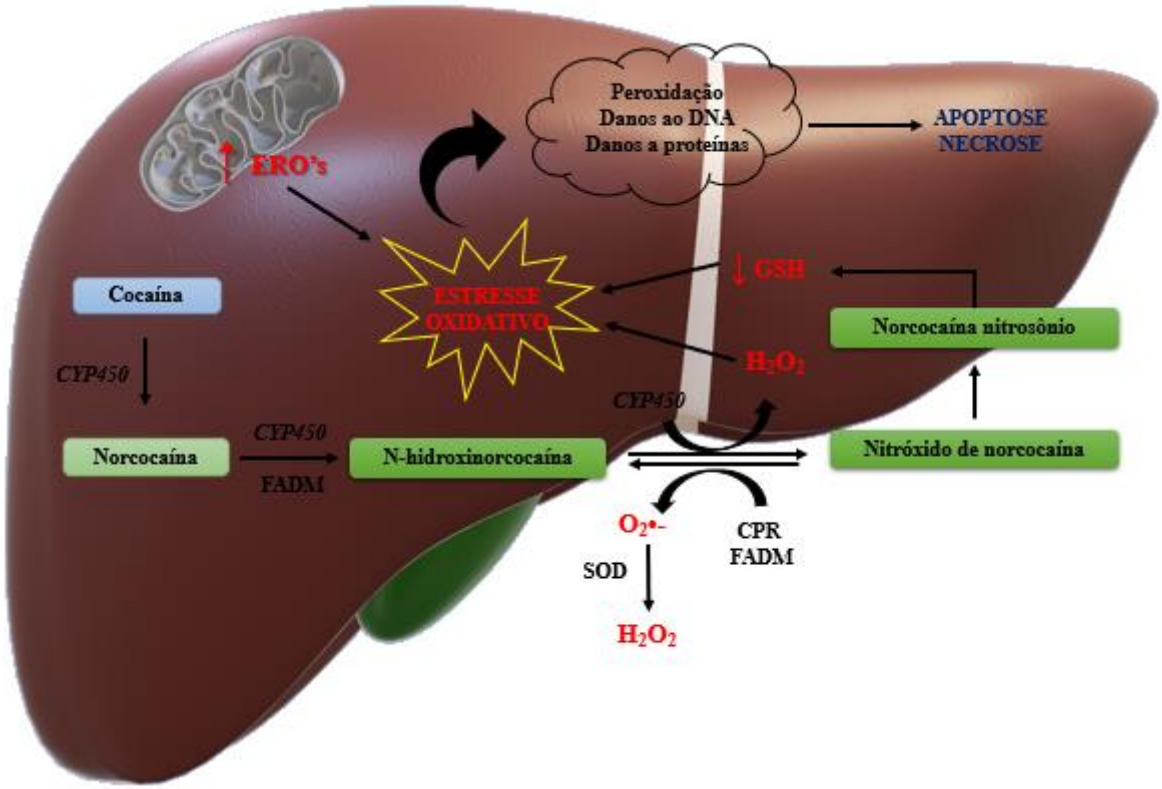

Figura 9 – Possíveis mecanismos de lesões hepáticas induzidas pela cocaína.
ERO's – espécies reativas de oxigênio; CYP450 – citocromo P450; FADM – flavina-adenina dinucleotídeo monooxigenases; CPR – citocromo P450 redutase; GSH – Glutationa reduzida; $O_2^{\bullet-}$ – íon superóxido; SOD – Superóxido Dismutase; H_2O_2 – peróxido de hidrogênio. Fonte: Adaptado de Valente *et al.*, 2012.

Em relação a nefrotoxicidade, parece que as consequências da cocaína ao tecido renal ocorrem, predominantemente, de uma forma indireta (**Figura 10**). Danos nas células renais por estresse oxidativo por ERO's geradas no próprio tecido não são descartadas, entretanto, o potencial metabólico das células renais é inferior quando comparado ao fígado. Esses mecanismos indiretos, como descritos anteriormente, parecem estar envolvidos com as alterações cardiovasculares, devido ao desenvolvimento de hipertensão renal, podendo causar sérios problemas renais (Mansoor *et al*., 2017).

Outro mecanismo indireto está associado com o efeito da cocaína sobre a musculatura esquelética, a qual pode haver uma intensa destruição de fibras musculares (rabdomiólise), com o extravasamento de mioglobina, que é extremamente nefrotóxica (Naughton, 2008; Dinis-Oliveira *et al.*, 2012).

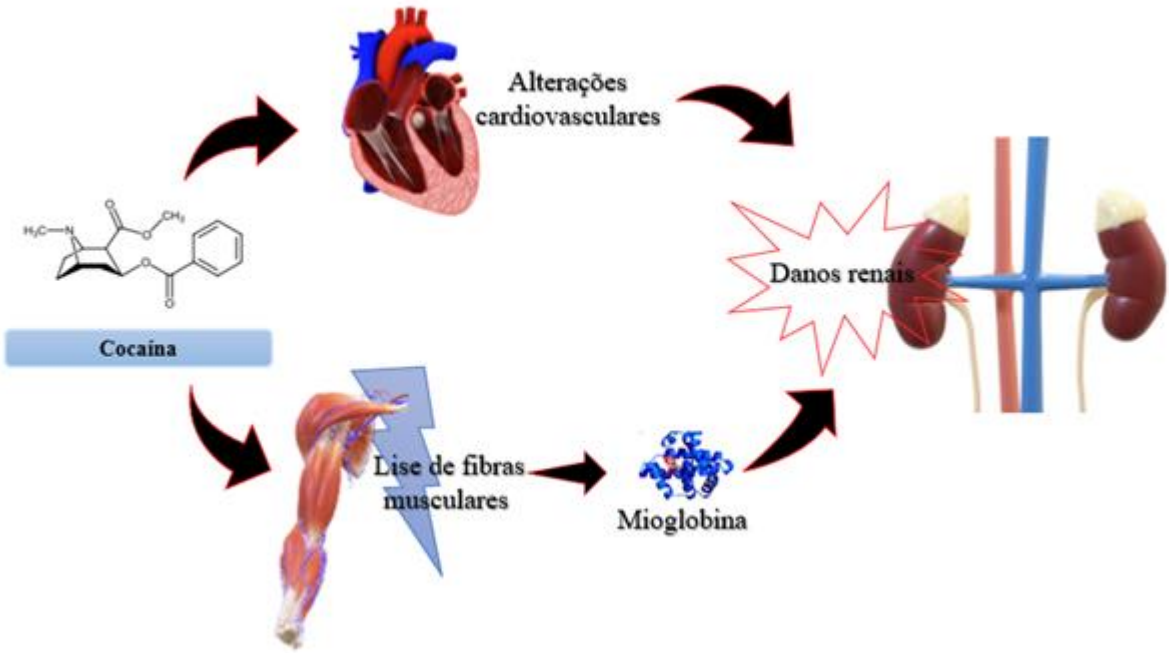

Figura 10 – Possíveis mecanismos indiretos de lesões renais induzidas pela cocaína.
Fonte: próprio autor.

1.7 Proliferação celular e Apoptose

Dois grandes momentos caracterizam o ciclo celular, a interfase e divisão celular, que são subdivididos em 4 estágios. A interfase é composta por 3 dos estágios: (i) fase G1 (gap 1), momento em que as atividades celulares visam o aumento do tamanho celular, preparando-a para replicação do DNA; (ii) fase S (síntese), onde ocorre a duplicação do material genético (replicação); (iii) fase G2 (gap 2), preparação da célula para a divisão celular. O último estágio corresponde a etapa da divisão celular, a fase M, na qual a célula divide-se originando células filhas (Rivoire *et al.*, 2001). A homeostase desse processo é garantida pela atuação de diversas proteínas, dentre elas, as quinases dependentes de ciclinas (CDKs), que regulam o ciclo celular. Há também, a atividade da proteína Ki-67, cuja expressão ocorre ao final de cada fase do ciclo celular, sendo muito utilizada como um biomarcador de atividade proliferativa (Barbosa *et al.*, 2003; Queiroz *et al.*, 2009; Goitia-Durán *et al.*, 2010; Menom *et al.*, 2019).

A apoptose, por sua vez, é um mecanismo de morte celular controlada, a partir de um processo de autodigestão, podendo ser iniciada por diferentes estímulos. Os estímulos externos são caracterizados pela indução da apoptose por meio de receptores específicos na membrana celular (receptores da morte), com a ativação da via extrínseca. Os estímulos internos são caracterizados por mecanismos de estresse celular, tais como lesão do genoma ou perturbações no ciclo celular que podem ativar a via intrínseca. Ambas as vias culminam com a ativação de um grupo de proteínas, denominadas de caspases (**Figura 11**). As caspases são enzimas presentes no citoplasma, sob a forma inativada (pró-enzimas) são conhecidas como proteína efetoras de morte (Parolin & Reason, 2001; Spencer Netto & Ferraz, 2001).

Na via extrínseca, a ativação de um dos receptores de membrana irá recrutar e ativar pró-caspase-8 e pró-caspase-10. Essas duas proteínas irão ativar pró-caspase-3, deflagrando a morte por apoptose (Grivicich *et al*., 2007). No processo da apoptose pela via intrínseca, a mitocôndria possui um papel fundamental, ao passo que essa organela armazena substâncias capazes de induzir a apoptose, como o citocromo C. Uma cascata de eventos irá culminar na apoptose, tal qual as proteínas pró-apoptóticas induzem a liberação do citocromo C ao citoplasma, que se associa a pró-caspase-9, tornando-a uma enzima ativa. A caspase-9, por sua vez, ativa as pró-caspases-3, que executa o processo propriamente dito (Parolin & Reason, 2001; Arruda *et al*., 2008).

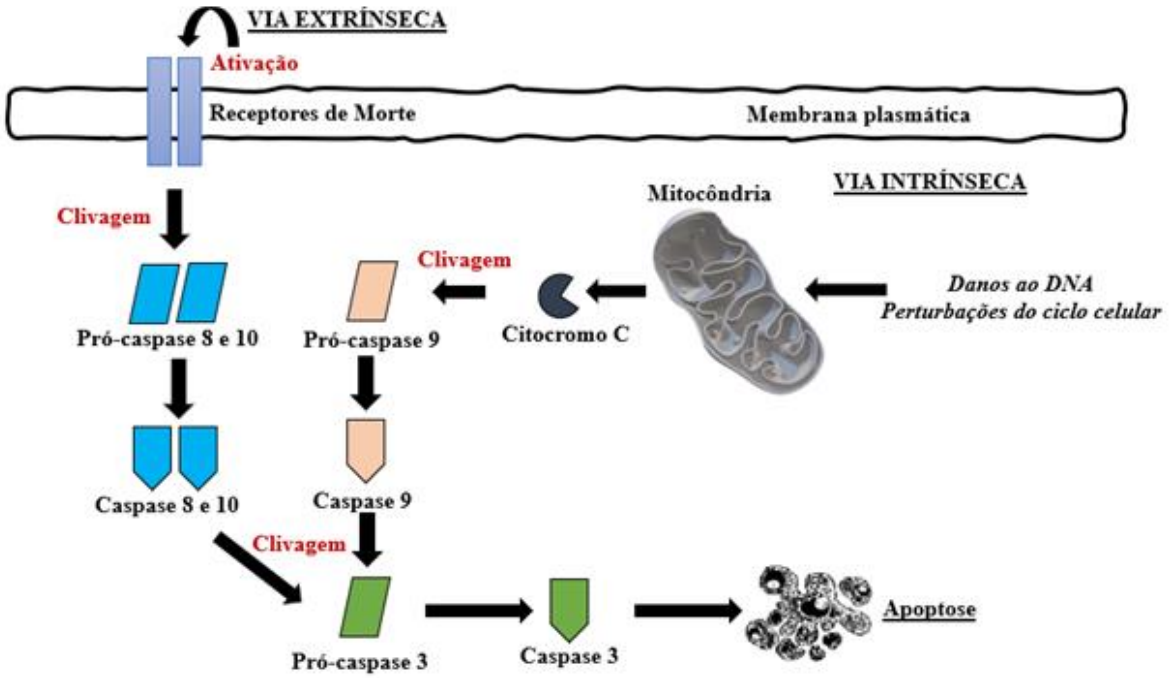

Figura 11– Representação esquemática das vias que culminam em apoptose.
Fonte: Próprio autor

1.8 Avaliação da Mutagenicidade pelo Teste do Micronúcleo

O teste do micronúcleo (MN) surgiu em meados dos anos 70, cuja sua finalidade é detectar danos cromossômicos, entretanto, apenas células com um potencial de divisão celular podem ser submetidas ao ensaio (Ballestreri, 2017). Atualmente, em mamíferos, o teste é comumente realizado em eritrócitos da medula óssea, entretanto, existem outros tipos celulares que também podem ser utilizados, como por exemplo, linfócitos do sangue periférico, hepatócitos e as células esfoliadas da mucosa bucal e nasal, com grande popularidade nos ensaios de biomonitoramento ambiental (Agostini, 1993; Uno *et al.*, 2015; OECD, 2016).

A presença de células micronucleadas, é reconhecida pela literatura, como um importante biomarcador sugestivo para o risco de câncer, pois reflete os primeiros eventos moleculares face a exposição de substâncias mutagênicas, haja visto, a mutação é um processo primordial no início da carcinogênese (Wunsch Filho & Gattas, 2001; Dong *et al.*, 2019).

Os MN são fragmentos de cromossômicos acêntricos ou cromossomos inteiros que não foram incorporados ao núcleo principal (**Figura 12**), sendo possível verificar sua presença após

a divisão celular, onde os mesmos estarão presentes nas células filhas. As substâncias consideradas mutagênicas são classificadas em agentes clastogênicos, que são aqueles que atuam na quebra dos cromossomos; e agentes aneugênicos, que interferem no fuso mitótico (Agostini, 1993; Carrard *et al.*, 2007; OECD, 2016).

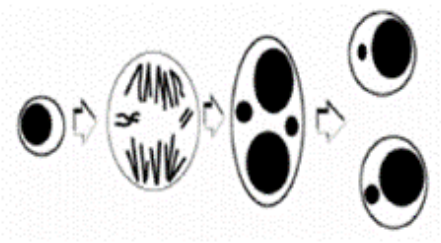

Figura 12 – Formação de células micronucleadas.
Fonte: Chequer, 2008.

Alguns critérios devem ser levados em consideração para, de fato, caracterizar a presença de micronúcleo nas células esfoliadas. Como dito anteriormente, apenas células passiveis de divisão celular podem ser submetidas ao ensaio. Outros critérios também são importantes na identificação do MN, tais como: i) possuir uma coloração de intensidade semelhante ou levemente mais fraca do que a do núcleo principal; ii) possuir uma borda bem delimitada, com a qual seja possível identificar a presença de uma membrana perinuclear; iii) dispor de uma morfologia arredondada; iv) estar presente no citoplasma da célula e separado do núcleo principal; v) conter um diâmetro de até 1/3-1/16 em relação ao núcleo principal. Ademais, as células em avaliação podem apresentar mais de um micronúcleo, entretanto, os mesmo devem possuir as características descritas acima (Carrard *et al.*, 2007; Reis *et al.*, 2002).

O teste do MN é uma técnica que vem sendo bastante utilizada para a investigação da segurança de diversas substâncias químicas. O teste possui fácil reprodutibilidade, e seu baixo custo contribuí diretamente na adoção desse ensaio mundialmente, sendo amplamente recomendo por agências regulatórias como a primeira escolha nos ensaios de mutagenicidade *in vivo* para diagnosticar o potencial mutagênico de diversos xenobióticos (Chequer, 2008; OECD, 1997).

Estudos anteriores realizados pelo nosso grupo de pesquisa, têm revelado que doses agudas de crack são capazes de induzir danos genéticos em múltiplos órgãos de roedores expostos ao crack (Moretti *et al.,* 2016, Yujra *et al.,* 2015). Em humanos, foi observada mutagenicidade em células esfoliadas da mucosa bucal de usuários de crack (das Graças Alonso de Oliveira *et al.,* 2014). Entretanto, não se sabe até o presente se o crack é capaz de induzir o aumento de micronúcleos em células hepáticas.

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CAPÍTULO II

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Genotoxicity, oxidative stress and inflammatory response induced by crack-cocaine: relevance to carcinogenesis

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Running title: genotoxicity induced by crack-cocaine

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Abstract

Crack-cocaine is a cocaine by-product widely consumed by general population in developing countries. The drug is low cost and is associated with more intense effects when compared to other illicit drugs. Genotoxicity, oxidative stress and inflammatory response are considered crucial events in carcinogenesis, since they actively participate in the multistep process. The purpose of this paper was to provide a mini review regarding the relationship between carcinogenesis and genotoxicity, oxidative stress and inflammation induced by crack-cocaine. The present study was conducted on search of the scientific literature from the published studies available in PUBMED, MEDLINE, Scopus and Google scholar for all kind of articles (all publications to November, 2020) using the following key words: crack-cocaine, DNA damage, genotoxicity, cellular death, cytotoxicity, mutation, oxidative stress, inflammation and mutagenicity. The results showed that published papers available were almost all in vivo test-system being conducted in humans or rodents. Crack-cocaine was able to induce genotoxicity and oxidative stress in mammalian cells. However, the role of inflammatory response after exposure to crack-cocaine was not conclusive so far. In summary, this study is consistent with the notion that crack-cocaine is a chemical carcinogen as a result of genotoxicity and oxidative stress induced in mammalian and non-mamallian cells.

Key words: crack-cocaine; genotoxicity; cytotoxicity; mutagenicity; oxidative stress; inflammation

1. Introduction

Nowadays, Brazil represents a big consumer of cocaine and other illicit drugs (Rosario et al. 2019). In particular, Brazil is the first consumer of crack-cocaine so far (UNODC 2014). The country is on the drug trafficking route from Latin America to Europe through ports and airports.

Crack-cocaine is a cocaine by-product poorly soluble in water through the presence of some chemical agents in its composition (Alexander 2006). Since Brazil is a developing country with a significant portion of population living in poverty or in extreme poverty, crack-cocaine has been widely consumed due to its low cost and more intense effects when compared to other illicit drugs (Ribeiro et al. 2006). It is well established that the underlying mechanisms behind crack-cocaine is dedicated to the brain through modulation of neurotransmitters and blocking dopamine reuptake (Morton 1999; Baik 2013). Thus, the biological risk of the drug is associated with euphoria followed by feeling of wellbeing. This inevitably leads to greater dependence (Dackis and O´Brein 2001).

Carcinogenesis is defined as a sequence of events required to normal cell undergoes malignant phenotype (Hanahan and Weinberg 2011). For this purpose, several steps are important and mandatory to trigger carcinogenesis, as follows: initiation, promotion and progression (Hanahan and Weinberg 2011). At the initiation phase, chemical agents are able to damage the genome of the cell (genotoxicity). After that, the injury will be permanently incorporated into the genome if the damage is not properly repaired (Angelieri et al. 2017). In the promotion phase, newly mutated cells are stimulated to proliferate (Angelieri et al. 2017). Multiplication of these ones will inevitably lead to tumor formation (progression phase). Genotoxicity participates in all steps of carcinogenesis, particularly in the initiation stage.

It is well discussed in literature that oxidative stress can activate a variety of pathways that leads to an oxidative imbalance damaging mammalian cells and, after extended periods,

promote carcinogenesis (Reuter et al. 2010). The increase of ROS may be due to endogenous oxidative stress, from hepatic metabolism and the action of the enzyme P450, mitochondria activities, NADPH enzymes, or exogenous origin, such as those generated by chemical substances (Klaunig et al. 1998; Uddin et al. 2019). ROS molecules are involved in carcinogenesis by gene mutations on injured cell or by its interference in transduction and/or transcription factors (Noda and Wakasugi 2001).

The close relationship between inflammation and cancer and many other disorders is also an important link in the development of potentially malignant diseases (Korniluk et al. 2017; Uddin et al. 2020). Inflammatory cells as well as pro-inflammatory cytokines frequently involve tumor area and its condition leads to neoplastic progression promoting successive oxidative DNA damage and decreasing its repair system (Korniluk et al. 2017). In fact, leukocytes are responsible for disrupting the redox system and they promote genomic instability (Korniluk et al. 2017). Some authors have shown inflammatory activity through leukocytes activation, coupled with decreased levels of nitric oxide (Albini et al. 2017). Taken together, it is reasonable to establish an association between carcinogenesis and genotoxicity, oxidative stress and inflammatory response induced by chemical agents, which are considered harzadous for the safety or health.

To our knowledge, there are no studies addressing new information on the relationship between carcinogenesis and genotoxicity, oxidative stress, inflammation induced by crack-cocaine in humans and other species. It should be noted that some international regulatory agencies, such as IARC and EPA are concerned in identifying chemical agents capable of participating directly or indirectly in carcinogenesis. Such information helps in the decision-making as well as in the protection of populations exposed to the potential harm. Thus, the aim of this study was to search the scientific papers published within the field. The study focused on four aspects: (i) the distribution of publications over the years (ii) the test-system adopted

in the study (human or other species) (iii) the main findings and (iv) the identification of gaps for developing future perspectives, focusing carcinogenesis.

2. Material and methods

2.1 Scientific literature search

The present study was conducted on search of the scientific literature from published studies available in PUBMED, MEDLINE, Scopus and Google scholar for all kind of articles (all publications to November, 2020) using the following key words: crack-cocaine, DNA damage, genetic damage, genotoxicity, mutation, and mutagenicity, inflammation and oxidative stress (**Figure 1**). No time limit was given in order to identify all papers published in the scientific literature. Nevertheless, review papers, case reports and papers not written in English language were not considered. After that, all manuscripts were identified by title, year of publication, type of experimental design (human or other species) and main findings.

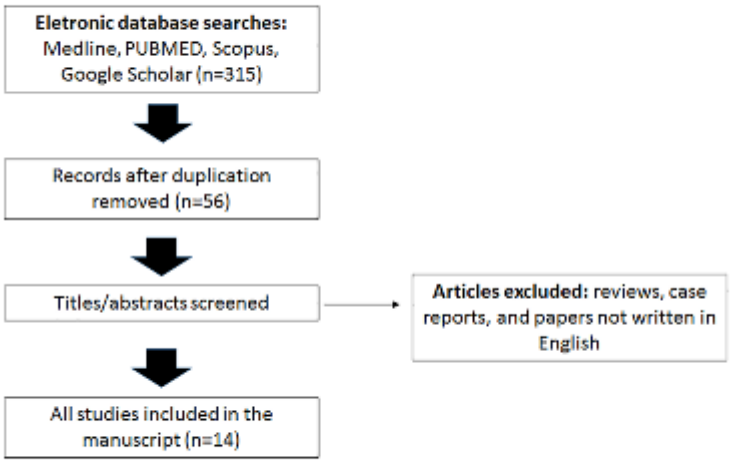

Figure 1. Design of the study.

3. Results

3.1 Genotoxicity

After searching the scientific literature, many papers were identified between 1997 and 2020. All articles are presented in **Table 1.** The majority of studies were conducted in humans in vivo.

Table 1. Publications regarding cytogenotoxicity induced by crack cocaine in alphabetical order of authors

Author(s)	Year of publication	Experimental Test system	Main findings
Albini et al.	2017	Human	Inflammation, microbial changes, increased cytoplasmic/nucleus ratio and descreased cytoplasmic volume
Almeida et al.	2012	Human	Chromosomal breakage and cellular death in oral mucosa cells
Antoniazzi et al.	2018	Human	Chromosome breakage and cellular death and oral lesions in the mouth
Das Graças Alonso de Oliveira et al.	2014	Human	Chromosomal breakage and cellular death in oral mucosa cells
De Freitas et al.	2014	Human	DNA damage and mutation in blood cells
Dos Santos Barbosa Ortega	2018	Mussel Perna Perna	DNA damage Modulation of GST expression
Garcia et al.	2015	Rat	Increased neurotoxicity and DNA strand breaks in hippocampal cells
Garcia et al.	2012	Rat	Cellular death and increased caspase 3 expression of hippocampal cells
Maranho et al.	2017	Mussel Perna Perna	DNA strand breaks and cellular death
Moretti et al.	2016	Rat	DNA damage in blood and mutation in bone marrow cells
Roth et al.	2004	Human	Decreased nitric oxide levels and bacterial killing performance from alveolar macrophages
Stefanidou et al.	2011	Protozoan Tetrahymena	Increased aneuploidy, disturbances in cellular activity and heterogeneity of chromatin

Stefanidou et al.	2002	Protozoan Tetrahymena	Increased DNA content
Stolf et al.	2014	Human	Polymorphism of DAT1 gene
Webber et al.	2016	Human	Chromosomal damage in oral mucosa cells
Yujra et al.	2016	Mouse	DNA damage in blood and brain cells

The vast majority of the scientific papers conducted in humans evaluated the biological effects on oral mucosa by using the single cell gel (comet) assay or micronucleus test. These asssays are able to detect DNA strand breaks and chromosome damage in mammalian cells, respectively. Seen from this perspective, some authors have demonstrated high number of micronucleated cells in exfoliated oral mucosa cells of crack-cocaine users attending a drug addiction treatment group at the International Federation of Red Cross in Porto Alegre (Brazil) (Webber et al. 2016). Moreover, some authors have also shown the presence of cell death (cytotoxicity) in buccal mucosa cells of individuals living in the streets from Brazilian cities (Almeida et al. 2012; das Graças Alonso de Oliveira et al. 2014). As previously mentioned in Introduction, such information is relevant to carcinogenesis, since DNA damage (genotoxicity) participates in the initiation phase of chemical carcinogenesis. Certainly, these data support the hypothesis that crack-cocaine induces genotoxicity on oral cells.

In order to support the results obtained to the oral mucosa, further efforts are needed to detect damage induced by crack-cocaine in other cell types. Using single cell gel comet and micronucleus assays, some authors were able to detect the presence of DNA injury and chromosomal breakage in peripheral blood cells from crack-cocaine users admitted for treatment at the drug rehabilitation centre in the city of Brazil (de Freitas et al. 2014). This means that crack-cocaine induces DNA strand breaks and mutations in peripheral blood cells as well.

Furthermore, genotoxicity seems to be supported by clinical studies that assumed a cause-effect relationship by increasing the incidence of oral neoplasms and pre-cancerous lesions in individuals continuously exposed to crack-cocaine (Antoniazzi et al. 2018; Cury et al. 2018). However, the incidence of periodontal disease in these volunteers is not conclusive so far (Antoniazzi et al. 2016; Cury et al. 2017). Taken together, it seems the crack-cocaine is a chemical carcinogen responsible for inducing genotoxicity in human cells.

In rodents, the results showed that genotoxicity was found after the exposure to crack-cocaine. Particularly, it was possible to identify what organs and/or tissues that are more sensitive to crack-cocaine with increasing doses during a short period of time. Moretti et al. (2016) have provided evidence that crack-cocaine is genotoxic in peripheral blood and bone marrow cells of rats. The authors found genotoxicity at 9mg/kg and 18mg/kg concentration. Yujra et al. (2016) have detected DNA damage in peripheral blood and brain cells exposed to crack-cocaine in increasing doses (9mg/kg and 18mg/kg) in vivo. Conversely, liver and kidney cells did not demonstrate any signs of genotoxicity after crack-cocaine exposure (Moretti et al. 2016; Yujra et al. 2016). Both studies have demonstrated the presence of cocaine of the tested substance. In vitro analysis has revealed some toxicity after exposure to anhydroecgonine methyl ester, a crack-cocaine pyrolysis product, by means of decreased number of viable cells (MTT assay), increased caspase 3 expression and DNA fragmentation in rat hypocampal and Chinese hamster ovary (CHO) cells in vitro (Garcia et al. 2012; 2015). Such effects were detected at concentrations ranging from 10^{-3} to 10 uM.

Other species have supported the hypothesis that crack-cocaine is genotoxic as well. For example, Mussel *Perna Perna* exposed in vitro to crack-cocaine demonstrated dose response relationship for DNA damaging, and cellular death (Maranho et al. 2017; Dos Santos Barbosa Ortega et al. 2019). Particularly, DNA damage was significantly increased in Mussels exposed

to 500 μg·L^{-1} of crack-cocaine (Maranho et al., 2017). The crack-cocaine used in this study had ~38% of cocaine. The protozoan Tetrahymena is also very sensitive to crack-cocaine as demonstrated by increased DNA content, decreased phagocytic activity, and increased aneuploidy in a dose dependent manner (Stefanidou et al. 1999; 2002; 2011). The results showed a decrease of up to 23% in the phagocytic activity of the protozoan Tetrahymena exposed to crack-cocaine at concentrations ranging from 0.5 to 2mg.

3.2 Oxidative stress

Regarding oxidative stress, it seems that crack-cocaine is a powerful oxidant agent on mammalian cells and other species. The results from literature search are presented in **Table 2.**

Table 2. Publications regarding oxidative stress induced by crack cocaine in alphabetical order of authors

Author(s)	Year of Publication	Test-system used	Main findings
Dos Santos Barbosa Ortega et al. 2019	2019	Mussel perna perna	Reactive oxygen species, denzofuran, glutathione levels were increased.,
Garcia et al.	2019	Hippocampal cells in vitro	Increased lipid peroxidation
Gomes et al.	2018	Rat	Increased oxidation protein products, thiobarbituric acid, and decreased catalase, glutathione peroxidase, and superoxide dismutase levels.
Lipaus et al.	2019	Rat	Increased lipid peroxidation, thiobarbituric acid-reactive species (TBARS) levels, and advanced oxidation protein products (AOPP), decrased the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)
Narvaez et al.	2013	Human	High brain-derived neurotrophic factor (BDNF) level.
Parcianello et al.	2018	Human	Increased oxidative stress
Sordi et al.	2014	Human	Increased brain-derived neurotrophic factor (BDNF) and thiobarbituric acid reactive substances (TBARS) levels.

Souza-Silva et al.	2020	Rat	Increased lipid peroxidation and latee closure of the fetal fontanel.
Zaparte et al.	2018	Human	Increased plasma levels of protein carbonyl, protein thiol content, and decrased superoxide dismutase (SOD), glutathione peroxidase (GPx), reduced reduced (GSH) and total reactive antioxidant potential (TRAP).

Recently, Souza-Silva et al. (2020) have demonstrated placental oxidative stress as a result of lipid peroxidation in rats, which smoked crack-cocaine. Moreover, other classical parameters closely related to teratogenesis were detected after exposure to crack-cocaine, such as abnormal fetal growth (Souza-Silva et al. 2020). The crack rocks used in this study had ~64% of cocaine. Certainly, such data show harmful effects of crack-cocaine on users and newborns exposed to the illicit drug. Other authors also have revealed the presence of lipid peroxidation since malonaldehyde (MDA) and thiobarbituric acid-reactive species (TBARS) levels. Advanced oxidation protein products (AOPP) were increased in some brain regions of rats exposed to crack-cocaine (Garcia et al. 2019; Lipaus et al. 2019). Following the same rationale, the levels of antioxant status were altered after exposure to crack-cocaine as depicted by decreasing glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) (Garcia et al. 2019). Other anti-oxidant enzymes were also decreased by crack-cocaine inhalation as for example superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Lipaus et al. 2019). The cocaine found in this sample was 26%. The same findings were verified by others (Gomes et al. 2018). Taken as a whole, it seems that crack-cocaine is able to induce oxidative stress as a result of lipid peroxidation.

In non-mamalian species, crack-cocaine also exerts some harmful effects closely related to oxidative stress. Such data are revelent mainly for ecototoxicity and environmental health. Dos Santos Barbosa Ortega (2019) have demmonstrated that *Mussels perna perna* has high levels of reactive oxidative species, followed by changes in the function of xenobiotics

metabolizng enzymes, such as glutatione transferases when exposed to crack-cocaine (Dos Santos Barbosa Ortega et al. 2019).

In humans, there are few studies within the field so far. Newborns exposed to crack-cocaine in utero have revealed the presence of oxidative stress during pregnancy (Parcianello et al. 2018). These data were collected from the historical archives of the Hospital. The oxidative stress was detected by thiobarbituric acid reactive substances (TBARS) levels in the plasma of crack-cocaine users recruited at admission to a public addiction treatment unit (Sordi et al. 2014) an when crack-cocaine was withdrawn (Sordi et al., 2020).

Addittionally, some atuhors have detected higher oxidative stress after short internal of non-exposure, i.e. 4 days. These findings were supported by protein carbonyl, protein thiol content, superoxide dismutase (SOD), glutathione peroxidase (GPx), reduced reduced (GSH) and total reactive antioxidant potential (TRAP) levels (Zaparte et al. 2015). Fortunately, the recovery for 18 days was effective to establish the oxidative status on mammalian cells as shown by higher levels of SOD, GPx, GSH and TRAP (Zaparte et al. 2015).

3.3 Inflammatory response

Among the cytokines that participate in the inflammatory process, IL-6 plays an important role in the acute phase. Furthermore, IL-8, also known as neutrophil chemotactic factor, induces chemotaxis, stimulates phagocytosis and angiogenesis in the acute plase of inflammatory process. In fact, some authors have demonstrated high expression of IL-8 and IL-6 in peripheral blood of crack-cocaine users from therapeutic communities in Southern Brazil (Levandowski et al. 2016). Crack-cocaine users also have revealed high levels of brain-derived neurotropic factor (BNDF), and higher circulating levels of IL-1B, TNF-alfa and IL-10 in peripheral blood cells when compared to matched controls (Narvaez et al. 2013). These findings are summarized in **Table 3.**

Table 3. Publications regarding inflammation induced by crack cocaine in alphabetical order of authors

Author(s)	Year of Publication	Test-system used	Main findings
Lewandowski	2016	Human peripheral blood	Higher circulating levels of IL-6
Narvaez	2013	Human peripheral blood	higher circulating levels of IL-1β, TNF-α and IL-10

Conclusion

The mini review was able to display the current scientific knowledge on the relationship between carcinogenesis and genotoxicity, oxidative stress and inflammatory response induced by crack-cocaine. The results showed that crack-cocaine is able to induce genotoxicity and oxidative stress in mammalian cells. In humans, it was possible to detect genetic damage and mutations in peripheral blood and oral mucosa cells from crack-cocaine users. Moreover, it was possible to identify that crack-cocaine is able to exert genotoxicity in multiple organs, such as peripheral blood, brain and oral mucosa in rodents (**Figure 2**).

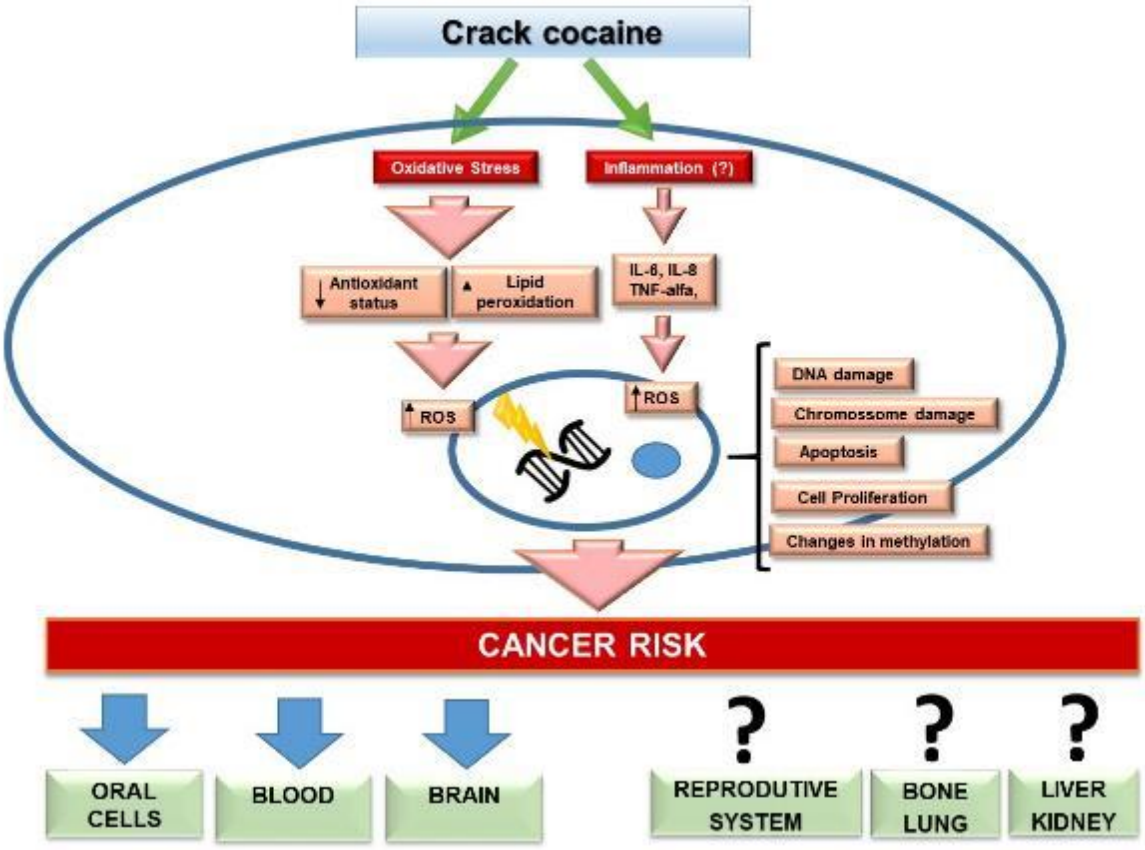

Figure 2. Crack cocaine as inducer of genotoxicity and oxidative in several cellular types.

Regarding oxidative stress, some biomarkers have revealed potential toxicity exerted by crack-cocaine. However, the role of inflammatory response after exposure to crack-cocaine was not conclusive so far. Therefore, new studies within the field are important for better understanding the underlying mechanisms of inflammatory response on human cells, especially to clarify what cells and/or tissues are more sensitive to crack-cocaine, as for example, upper respiratory tract, liver, heart and kidney (Figure 2). The elucidation of other cell signalling pathways linked to cell death, such as apoptosis or necrosis, DNA repair system and mutations are welcomed. In another context, the potential efficacy of polyphenols as for example, epigallocatechin-3-gallate, curcumin, resveratrol, quercetin against the toxicity induced by crack cocaine may be a promising tool in non-fatal cases of severe acute systemic intoxication (Hannan et al. 2020; Uddin et al. 2020b).

Another question concerns to the chemical composition of crack-cocaine. The cocaine was present in all samples evaluated in these studies, but its concentration ranging from 20-67%. This does not allow a more precise comparison between studies, as well as to evaluate the real impact of cocaine on individuals exposed to crack-ococaine.

Finally, it is important to stress that quality of life is severely impacted in crack-cocaine users, especially in terms of general, mental and physical health. Moreover, alcohol abuse, tabacco smoking, and malnutrition commonly observed in crack-cocaine users (Coelho et al. 2020; Knerich et al. 2019; Abreu et al. 2018). Certainly, these risk factors may increase free radicals and inflammation and decrease antioxidant defenses in chronic users contributing to the cancer risk. Also, there is a higher risk of death by fatal overdose, suicide or cardiovascular events rather than cancer-associated mortality in crack-cocaine users (Gicquelais et al. 2020). Taken as a whole, these features are potential confounding factors, when investigating co-morbities in crack cocaine users. In this regard, further follow-up studies are mandatory to evaluate the relationship between crack-cocaine and human cancer development.

In summary, this study is consistent with the notion that crack-cocaine is a chemical carcinogen as a result of genotoxicity and oxidative stress induced in mammalian and non-mammalian cells.

Ethics approval and consent to participate

Not aaplicable.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Conflict of Interest

All authors declares that no conflict of interest.

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Authors' contributions

ITM, DVS, DAR and BAR made the literature search. MBV, CDSP, DE and DAR performed data interpretion. All authors wrote the manuscript.

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CAPÍTULO III

Artigo publicado na revista ***International Journal of Enviornmental Health Research.***

Histopathological and inflammatory response in multiple organs of rats exposed to crack

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ABSTRACT

The aim of the present study was to investigate histopathological and inflammatory response in liver and kidney of rats after crack exposure. For this purpose, a total of 32 male Wistar rats were distributed into four groups: (G1) and (G2): received 18 mg/kg of body weight (b.w) of crack cocaine, but Group G2 remained 72h without exposure after the experimental period (5 days). Experimental group 3 (G3): received 36 mg/kg of body weight (b.w) of crack cocaine. Control Group (CTRL): received only the vehicle (DMSO) administered by intraperitoneal (i.p) route for 5 days. The results showed that crack cocaine induced histopathological changes in liver and kidney. Immunohistochemistry data revealed that G2 group showed a higher immunoexpression of Ki-67 in hepatic and renal tissues. Regarding inflammation, the results showed that all groups exposed to crack cocaine decreased the expression of TNF-α, IL-6, and IL-10 in liver and kidney. In summary, our results showed that the subacute doses of crack cocaine used in this study had cytotoxic, and immunosuppressive effects in liver and kidney of rats, especially at 36 mg/kg dose. Since cellular death and inflammation participates in the multi-step process of chemical carcinogenesis, these data offer new insights into potential ways to understand the pathobiological mechanisms induced by crack cocaine in several tissues and organs.

Key-words: crack cocaine; cytotoxicity; inflammation; kidney; liver

1. Introduction

Crack is cocaine in its most harmful form, due to the presence of remnants of other chemical agents (adulterants), which are used during the refining process (Riezzo *et al.* 2012). The name crack is used as an onomatopoeic expression that refers to the crackling sound generated during pyrolysis, when the drug is consumed by smoking (Oliveira & Dinis-Oliveira, 2018). Considered one of the most used illegal drugs around the world, crack cocaine looks like irregular crystals in the form of "stones," of brownish and/or yellowish beige color (Andrade Filho *et al.* 2013).

Crack cocaine appeared in the mid-1980s and it was first identified in the United States (USA) (Bastos; Bertoni 2014). In 1996, crack cocaine users were profiled as, in most cases, young men with a low level of education and no formal employment. Most consumers use this drug for a long time, leading to physical, psychological and financial exhaustion. The craving for crack cocaine is responsible for the loss of interest in such essential activities as sleep, food and a sense of responsibility. In particular, financial issues can lead crack cocaine users to participate in illegal activities like trafficking, robbery or theft (Oliveira & Nappo 2008).

Inflammation is an essential mechanism that ensures organism protection against biological, physical and chemical agents, such as narcotics in general. It is known that inflammation also acts in tissue regeneration. Once tissue integrity is restored, inflammatory infiltrate ends. However, if there is any deregulation, inflammation remains, turning into a chronic process (Oishi & Manabe 2018). Cytokines are classified as pro- or anti-inflammatory activities. Tumor necrosis factor alpha (TNF-α), interleukin-1 beta and interleukin-6 (IL-1β and IL-6) activates inflammation, while interleukin-10 (IL-10) has anti-inflammatory activity (Souza *et al.* 2010).

It has been documented that cocaine-based drugs of abuse can affect the immune system by changing the levels of cytokines participating in the inflammatory process (Yamada &

Nabeshima 2004). Nevertheless, studies investigating inflammatory activity due to crack cocaine exposure are scanty. Narvaez *et al.* (2013) found plasma elevation of IL-1β, TNF-α and IL-10 in a study with humans exposed to crack cocaine and cocaine hydrochloride. In another study with humans, Gan *et al.* (1998) found decreased IL-10 plasma levels after exposure to cocaine hydrochloride. On the other hand, Irwin *et al*. (2007) found decreased IL-6 plasma concentration in cocaine hydrochloride-addicted individuals after two days of exposure. However, it is important to stress that the behavior of cytokines participating in inflammation upon crack cocaine exposure in other tissues and/or organs, such as liver and kidney, is still unknown. Certainly, some injuries, associated with the frequency with which they occur as a result of consumption of this drug, vary according to dose and individual susceptibility. This is because different tissues may present different adaptive responses (Yujra *et al.* 2015). Given these consequences, crack cocaine can generate several tissue injuries, since the drug is full of adulterants, besides cocaine. In fact, some researchers assumed that crack cocaine is able to exert more deleterious effects when compared to cocaine hydrochloride (Oliveira & Dinis-Oliveira 2018). Anyway, the liver and kidneys are the main organs of xenobiotics metabolism and excretion and, therefore, they are considered critical organs when investigating chemical agents, especially those considered suspect (Sales & Foresto, 2020).

The aim of this study was to investigate cytotoxicity and inflammatory response induced by crack cocaine exposure in liver and kidney of rats.

2. Material and Methods

2.1 Animals and Experimental Design

Thirty-two male Wistar rats (*Rattus norvegicus*) with mean weight of 250 g and 9 weeks of age were used to this study. They were obtained from the Center for the Development of Experimental Models for Medicine and Biology (CEDEME) at the Federal University of São Paulo – UNIFESP. The animals were kept in the Animal Facility of Department of Biosciences at UNIFESP in a 12-hour light-dark cycle and at room temperature of 22ºC ± 1ºC, with free access to water and commercial feed.

The animals were distributed into four groups with 8 animals in each group: Control (CTRL); Experimental (G1); Experimental-withdrawn group (G2); and Experimental (G3). G1 and G2 received 18 mg of crack/kg of body weight. Crack cocaine was diluted in dimethyl sulfoxide (DMSO) and administered (ip) once a day for 5 consecutive days. However, G2 remained 72 hours without administration of the drug before euthanasia after the same period established in Group 1. G3 received 36 mg of crack cocaine/kg of body weight, also diluted in dimethyl sulfoxide (DMSO) and administered (ip) once a day for 5 consecutive days. CTRL group received only the vehicle (DMSO), administered (ip) once a day for 5 consecutive days.

The crack cocaine doses used correspond to 25% (18 mg) and 50% (36 mg) of the lethal dose of 50% cocaine (LD50) in rodents (Salvadori *et al.*, 1998). To standardize the quantity to be injected, the final volume used in the vehicle was 1 mL/kg for all animals. The drug was given as a courtesy by the Criminal Department of Limeira City, Sao Paulo State, Brazil for research purposes.

After completing the established experimental periods, the animals were euthanized with a mixture of ketamine (75 mg/kg), xylazine (10 mg/kg), fentanyl (0.5 mg/kg) and acepromazine (1 mg/kg), administered in the same syringe (IP) to achieve a deep state of anesthesia, before cardiac exsanguination (euthanasia). No animals died during the experiment.

2.2 Chemical Analysis

The crack cocaine sample used in this study was chemically evaluated using mass spectrometry in a previous study conducted by our research group, identifying 37.99% of cocaine for every 100 mg of crack cocaine (Maranho *et al.* 2017). In addition, cocaine was detected as the main component of crack cocaine (Moretti *et al.* 2016).

2.3 Histopathological Analysis

Once tissue fixation was complete, liver and kidney were gradually dehydrated in alcohol, followed by xylene and later incorporated in paraffin blocks. The tissues were cut into 3-µm thick sections. The slides were deparaffinized in xylene and rehydrated in ethanol (99.5%), and then stained with hematoxylin-eosin (HE) for histopathological evaluation under a Zeiss-Axio Observer D1 microscope. Liver tissue assessment was made according to Zhang *et al*. (2006). Renal tissue evaluation was made according to the criteria adopted by Jiang *et al*. (2009), considering renal cortex injury severity, using a semi-quantitative scale. The following criteria were considered: inflammatory process, degenerations, and necrotic areas. These findings were scored, considering renal cortex injury severity.

2.4 Immunohistochemistry

For the immunohistochemical analysis, we investigated Ki-67 and cleaved caspase-3-expressions. All antibodies (Ki-67, Biocare Medical, USA, and cleaved caspase-3, Santa Cruz Biotechnology, USA) were used at a concentration of 1:150 for 24h. After that, sections were treated with universal biotin-conjugated secondary antibody (Starr Trek Universal HRP Detection, Biocare Medical, USA) at concentration 1:100 for 30 min, followed by the application of DAB (3,3-diaminobenzidine) at 0,05% (DAKO® North America, Inc®,

California, EUA) and conterstained with hematoxylin for cleaved caspase-3 or fast green for Ki-67. The immunohistochemical evaluation was performed according to the antibody used: *Anti-Ki-67*: protein expression was analyzed by a semi-quantitative method, with the frequency of positive nuclei counted from 1,000 cells, according to Zhou *et al.*, (2006); *Anti-Caspase-3-cleaved*: protein expression was evaluated by a semi-quantitative method according to Salim *et. al.*, (2008), adopting the criteria of labeling extent by scores: no labeling (0); <10% labeling extent (1); 10-50% labeling extent (2), and > 50% labeling extent (3).

2.5 Expression of inflammatory and anti-inflammatory proteins in liver and kidney tissue

Inflammatory (TNF-α; IL-6; IL-1β) and anti-inflammatory (IL-10) cytokines were analyzed by the enzyme-linked Immunoabsorbent Assay (DuoSet ELISA) of capture (R&D System, Inc., Minneapolis, USA). In this purpose, total proteins were extracted from the samples. For this, central fragment from liver and kidney was homogenized in lysis buffer (10 mM EDTA, 100 mM Tris-HCl, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 2 mM phenylmethylsulphonyl fluoride, 0.1 mg bovine lung aprotinin, 1% Triton) and centrifuged at 20,000 RCF for 40’ at 4ºC. The supernatant was collected, and the protein content was assessed with Bradford reagent. Interleukin 6, 10, 1β, and TNFα protein content were determined by the enzyme-linked immunosorbent assay (ELISA) method (R&D System, Minneapolis, MN, EUA) following the manufacturer's instructions. Quantification of inflammatory (TNF-α; IL-6; IL-1β) and anti-inflammatory (IL-10) cytokines was performed using commercial kits (DuoSet ELISA, R&D Systems®, Minneapolis, MN, USA) using the Sandwich ELISA assay. In this, 96 well polystyrene microplates were incubated with capture antibodies (R&D Systems®). Afterwards, the last washing process was carried out.

2.6 Statistical analysis

The results were expressed as mean ± standard deviation (SD). Kruskal-Wallis non-parametric test was used for histopathological and immunohistochemical evaluation, followed by Dunn's post-test. In the ELISA assay, one-way Analysis of Variance (ANOVA) was used, followed by Tukey's multiple comparison post-test for both tissues (liver and kidney). $p < 0.05$ was considered as significant statistically. All statistical analyses were performed using Graph Pad Prism™, version 6.0.

3. RESULTS

3.1 Histopathological evaluation

Histopathological evaluation was performed in liver and kidney of animals exposed to subacute doses of crack cocaine for 5 days. The severity of the histopathological changes found was categorized by scores as shown in **Table 1**.

Table 1. Histopathological changes in liver and kidney of rats exposed to crack

Liver

Groups	**N**	**SCORE**			
		0	**1**	**2**	**3**
CTRL	8	**8**	0	0	0
G1*	8	**4**	**3**	**2**	0
G2**	8	**4**	**4**	0	0
G3*	8	**2**	**5**	**1**	0

Kidney

Groups	**N**	**SCORE**				
		0	**1**	**2**	**3**	**4**
CTRL	8	**8**	**0**	0	0	0
G1*	8	**5**	**2**	**1**	0	0
G2	8	**3**	**3**	**2**	0	0
G3*	8	0	**2**	**2**	**3**	**1**

Control Group (CTRL); Crack 18mg/kg group (G1); Crack 18mg/kg and 72h of abstinence group G2); Crack 36 mg/kg group (G3). ***p<0.05 when compared to control group (CTRL).**p<0.05 when compared to G1.

In hepatic tissue, the animals from CTRL group showed normal tissue structure, characterized by distinct liver cells, uniform nuclei, sinusoidal spaces and centrilobular vein. However, animals from G1 and G3 presented some foci of coagulative necrosis, and tissue disorganization (**Figure 1A-D**). The withdrawn group (G2) was able to reverse tissue injury induced by crack cocaine. The scoring reached through comparisons of the groups demonstrated significant statistically differences ($p < 0.05$) between G1 and G3 groups when compared to CTRL group.

Regarding kidney tissue, its morphological structure was preserved in CTRL animals, while G1 and G3 animals had extensive areas with coagulation necrosis as a result of degenerated tubules, and increased eosinophilia (**Figure 1E-H**). G3 animals showed more severe microscopic changes as compared to G1, but no significant statistically differences ($p>0.05$) were noticed between groups. In G2, there was no reversion of the tissue injury caused by crack cocaine in renal tissue, without significant statistically differences in relation to G1.

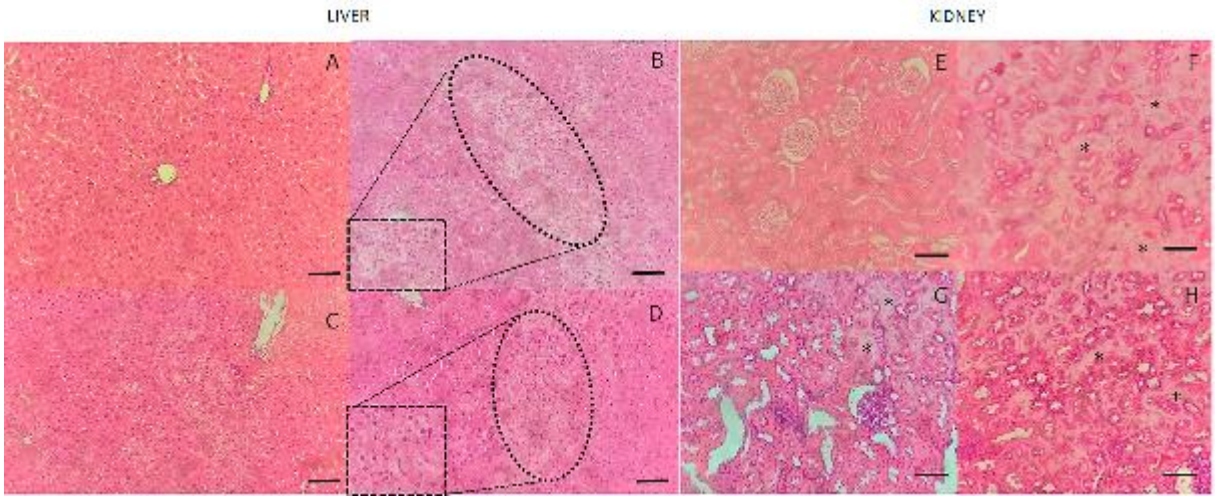

Figure 1. Photomicrographs of rat liver exposed to crack. Liver
A) Control group (CTRL); **B)** Crack 18mg/kg group (G1); **C)** Crack 18mg/kg and 72h of wtihdrawn (G2); **D)** Crack 36 mg/kg group (G3). Circle indicates coagulation necrosis with increased eosinophilia. Kidney: **E)** Control group (CTRL); **F)** Crack 18mg/kg group (G1); **G)** Crack 18mg/kg and 72h of withdrawn (G2); **H)** Crack 36 mg/kg group (G3). Note the presence of extensive coagulation necrosis, especially at 18 mg/kg dose. Asterisk indicates coagulation necrosis. **H.E. stain. Bar = 36µm.**

3.2 Immunohistochemical evaluation of proliferative and pro-apoptotic activities

In the hepatic and renal tissue, ki-67 immunoexpression was observed in the nuclei of hepatocytes and renal cells. All groups showed Ki-67 immunoexpression. In hepatic tissue, G1 increased proliferative activity as compared to CTRL (p<0.05). G2 showed higher frequency of immunoreactive cells in the liver tissue, with significant statistically differences (p<0.05) when compared to G1. In renal tissue, G1 and G3 also showed greater Ki-67 immunoexpression with significant statistically differences (p<0.05) in relation to CTLR. The withdrawn group (G2) showed increased proliferative activity when compared to G1, with statistically significant differences between groups (p<0.05). These results are demonstrated in **Figure 2.**

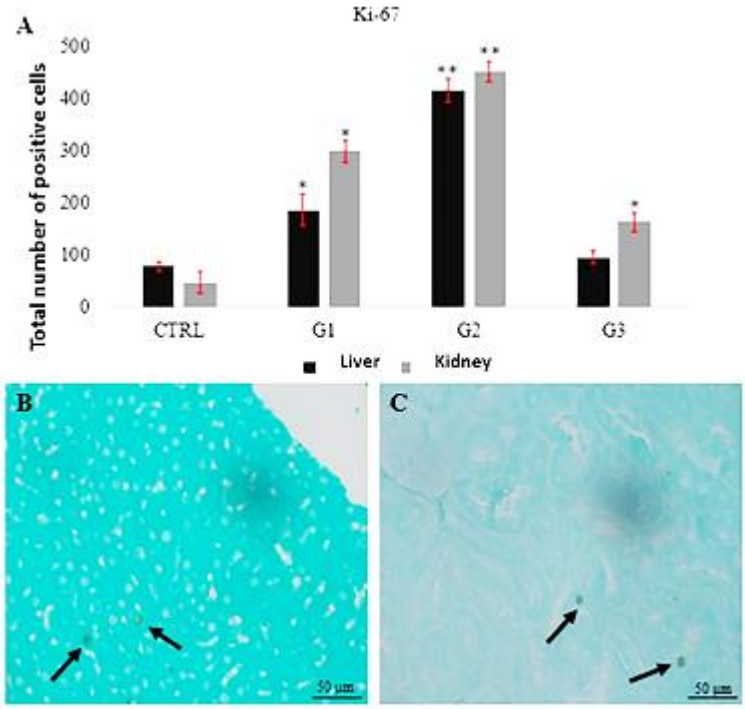

Figure 2. Immunohistochemistry for ki-67 in liver and kidney of rats exposed to crack cocaine. **A**) Total number of ki-67 imunopositive cells in liver and kidney of rats (mean + S.D.). Control group (CTRL); Crack 18mg/kg group (G1); Crack 18mg/kg and 72h of withdrawn (G2); Crack 36 mg/kg group (G3). **B**) Photomicrography of rat liver showing nuclear staining **C**) Photomicrography of rat kidney showing nuclear staining. *p<0.05 when compared to CTRL group. **p<0.05 when compared to G1.

Cleaved caspase-3 immunoexpression was detected in cytoplasm of cells. The numerical results are shown in **Table 2.** In liver tissue, G1 showed a significant statistically differences in relation to CTRL (p<0.05). G2 and G3 showed no differences in relation to their respective controls. Likewise, when the withdrawn group (G2) was compared with its respective control (G1), there was no significant statistically differences (p>0,05) between groups. Regarding kidney tissue, the same situation occurred, that is, only G1 showed statistically significant differences (p <0.05) when compared to CTRL.

Table 2. Immunohistochemical findings of cleaved caspase 3 in liver and kidney of rats exposed to crack

Liver					
Groups	**N**	**SCORE**			
		0	**1**	**2**	**3**
CTRL	8	**3**	**4**	**1**	**0**
G1*	8	0	**1**	**5**	**2**
G2	8	0	**4**	**2**	**2**
G3	8	**3**	**2**	**1**	**2**
kidney					
Groups	**N**	**SCORE**			
		0	**1**	**2**	**3**
CTRL	8	**3**	**3**	**1**	**1**
G1*	8	0	**0**	**5**	**3**
G2	8	0	**4**	**2**	**2**
G3	8	**3**	**2**	**1**	**2**

Control Group (CTRL); Crack 18mg/kg group (G1); Crack 18mg/kg and 72h of abstinence group G2); Crack 36 mg/kg group (G3). ***p*<0.05 when compared to control group (CTRL).

3.3 Inflammatory assessment

In hepatic tissue, either pro-inflammatory (TNF-α, IL-6 and IL-1β) or anti-inflammatory (IL-10) cytokines were present for all groups. The results showed a significant decrease (p<0.05) of TNF-α for all groups exposed to crack cocaine, regardless of the dose used. The same occurred for IL-6 and IL-10, since significant statistically differences (p<0.05) were detected in these groups as compared to CTRL. Regarding IL-1β, only the group exposed to the highest dose of crack cocaine (36 mg/kg) showed decreased expression for this marker as

compared to CTRL (p<0.05). In all cases, the withdrawn (G2 group) was not able to change the inflammatory expression when compared to G1. These results are shown in **Figure 3**.

In kidney, the analysis of inflammatory markers showed similar findings to those found for liver tissue. Regarding TNF-α, IL-10 and IL-1β, all groups exposed to crack cocaine at doses of 18 and 36 mg/kg showed a decreased expression as compared to CTRL (p <0.05). For the withdrawn group (G2), the results did not reveal any significant differences as compared to G1. However, it is important to note that the decrease in cytokine IL-6 occurred only in groups G2 and G3 as compared to CTLR. Finally, a clear dose-response effect was observed in decreasing IL-6 and IL-10 expression, while significant statistically differences were observed in groups G1 and G3 (p <0.05). **Figure 3** shows these results.

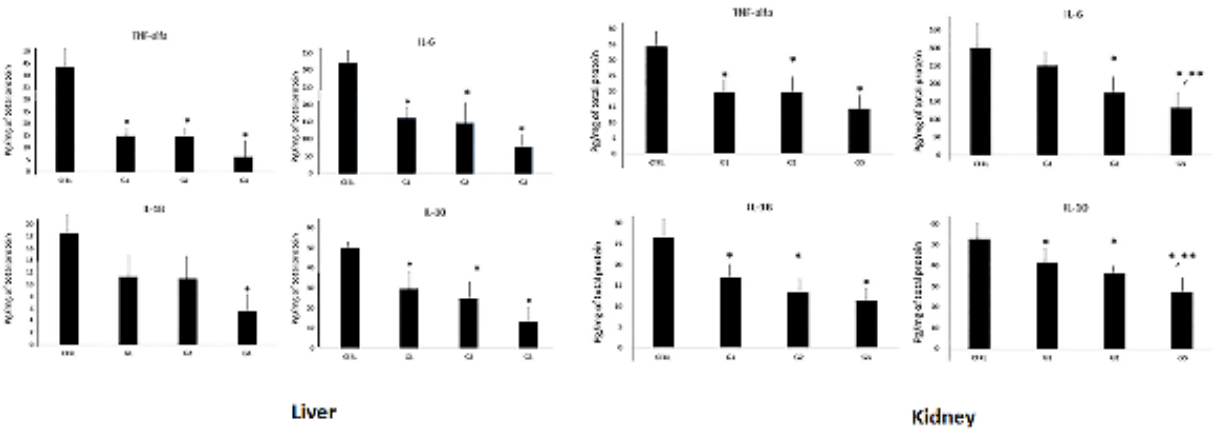

Figure 3. Expression of pro-inflammatory (TNF-α, IL- 6 e IL-1β) and anti-inflammatory (IL-10) cytokines in rat liver and kidney exposed to crack.
Control group (CTRL); Crack 18mg/kg group (G1); Crack 18mg/kg and 72 h of withdrawn (G2); Crack 36 mg/kg group (G3). *p<0.05 when compared to CTRL. **p<0.05 when compared to G1.

4. Discussion

Crack cocaine consumption is associated with several psychological changes, which are aggravated by the concomitant use of other psychoactive substances (De Queiroz *et al.* 2019). The high prevalence of crack cocaine consumption in society justifies studies for better understanding the putative pathobiological mechanisms induced by crack cocaine with respect to cytotoxicity and inflammatory activity.

The histopathological evaluation showed that liver and kidney of rats presented morphological changes in both doses used (18 mg/kg and 36 mg/kg), with significant statistically differences ($p<0.05$) in relation to control group, presenting several areas of coagulation necrosis in the liver parenchyma and renal cortex. In a previous published study, acute exposure (24h) to crack cocaine (18 mg/kg) was not able to induce histopathological changes in liver and kidney of rats (Moretti *et al.* 2016). However, when the hepatotoxic potential of cocaine hydrochloride at different doses and periods of time was investigated, the administration of cocaine hydrochloride (50 mg/kg) induced glycogenic degeneration and steatosis (Shuster *et al.* 1977). When the dose of cocaine hydrochloride was increased to 100 mg/kg, extensive necrosis in liver was evident after 24 hours of exposure. At a dose of 50 mg/kg, a liver regeneration was detected after 3-5 days of administration (Shuster *et al.* 1977). In a study conducted by Powers *et al.* (1992), liver exposed to cocaine hydrochloride at doses of 10, 20 and 30 mg/kg (i.p.) for 5 days showed results similar to those of Shuster *et al.* (1977) in mice.

Some authors investigated the effects of cocaine hydrochloride in kidney, in which rats received the drug (30 mg/kg, ip) once a day for 7, 15, 30, 45, 60, 75 and 90 days. Histopathological evaluation showed glomerular and tubular damage, as well as glomerular atrophy, edema, necrotic areas, and hemorrhage from day 15 on (Barroso-Moguel *et al.* 1995). In a study with mice, Shi *et al.* (2012) observed necrotic areas in liver and changes in the

biochemical profile of lipids and serum transaminases in animals that received cocaine hydrochloride (30 mg/kg, ip) once a day for 3 days.

In humans, there are few studies investigating crack cocaine toxicity in hepatic and renal tissue so far. Some case reports can be found in the scientific literature, which describe degeneration and necrotic processes in hepatic and renal tissues, emphasizing the correlation between renal injury and rhabdomyolysis, due to the massive destruction of muscle fibers (Dinis-Oliveira *et al.* 2012; Goel *et al.* 2014). Anyway, our results are fully in line with these findings (Alvarez et al., 1999; Vidyasankar et al. 2015). Our experimental group that remained 72 hours without crack cocaine administration (withdrawn), after 5 days of treatment with 18 mg/kg (G2), showed liver regeneration. These results are similar to the findings of Shuster *et al.* (1977). However, no signs of tissue regeneration were detected to kidney exposed crack cocaine.

In the present study, we evaluated cleaved caspase-3 expression by immunohistochemistry. All experimental groups showed immunoexpression of this marker in liver and kidney. In both tissues, there was a significant statistically difference in the groups exposed to crack cocaine at 18 mg/kg dose (G1). Recent studies have demonstrated high expression of cleaved caspase-3 in liver and kidney of mice (Western Blotting) after 6h and 24h of exposure to a single dose of 60 mg/kg of cocaine hydrochloride (Mai *et al.* 2016; 2018). In vitro study pointed out increased expression of cleaved caspase-3 (Western Blotting) after exposure to cocaine hydrochloride (Zaragoza *et al.* 2000; 2009). Our results are consistent with the above-mentioned studies. However, it should be noted that the withdrawn group (G2) was not able to modulate the apoptosis process, in liver and kidney of crack cocaine-exposed rats. Likewise, the highest dose of crack cocaine did not increase the cleaved caspase-3 expression. This is probably explained by the fact that the type of cell death that occurs in this group was predominantly by necrosis However, the triggering factors of apoptosis or necrosis are still a

matter of debate, although it is believed that necrosis would surpass apoptosis when the stimulus is of high magnitude.

Regarding proliferative activity, G1 showed greater Ki-67 immunoexpression in liver and kidney, with significant statistically difference (p <0.05) as compared to CTRL. In G3, only the renal tissue showed greater Ki-67 immunoexpression, with significant statistically difference (p <0.05) as compared to CTRL. Regarding G2, liver and kidney showed an increase in the proliferative rate as compared to G1. Thus, it was evident tissue regeneration in liver parenchyma, after drug withdrawal. Indeed, Shuster *et al.* (1977) showed liver tissue regeneration after a period of 3-5 days.

In the present study, we also evaluated the expression of pro-inflammatory (TNF-α, IL-6 and IL-1β) and anti-inflammatory (IL-10) cytokines in liver and kidney. In both tissues, we observed a decrease in TNF-α, IL-6, IL-1β and IL-10 in all crack cocaine-exposed groups, for all doses investigated. Some studies in humans and experimental animal models demonstrate an increase in pro-inflammatory cytokines, whereas others demonstrate a decrease in these cytokines (blood and brain). In addition, some studies correlate a lesser activity of immune cells, in rodents and humans exposed to crack cocaine (Irwin *et al.* 2007; Narvaez *et al.* 2013; López-Pedrajas et al. 2015). Taken as a whole, we assume that crack cocaine is a potent agent able to induce immunosuppressive effect in liver and kidney of rats.

One important limitation of the study refers to the experimental design since crack cocaine was administered by intraperitoneal injection. As it is well known, crack cocaine is consumed by smoking, which characterizes an environmental exposure through the lungs. In order to achieve more valid results, especially to establish the putative relationship between inflammatory response and cytotoxicity induced by crack cocaine in multiple organs, new examination is mandatory and planned by the authors. Even so, our results are very relevant to crack cocaine users, particularly because these people are continuously exposed to

other environmental conditions such as, tobacco smoking, alcohol abuse, and malnutrition (Coelho et al. 2020).

In summary, our results showed that the subacute doses of crack cocaine used in this study had cytotoxic, and immunosuppressive effects liver and kidney of rats, especially at 36 mg/kg dose. Since cellular death and inflammation participates in the multi-step process of chemical carcinogenesis, these data offer new insights into potential ways to understand the pathobiological mechanisms induced by crack cocaine in several tissues and organs.

Ethics approval and consent to participate

All procedures were conducted according to the International Research Standards for Animals, and the study was approved by the Ethics Committee on Animal Use (CEUA) of Federal University of Sao Paulo – UNIFESP, under Protocol no. 7038080219.

Consent for publication

Not applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DVS, BAR and BPC performed the experimental design. DVS and BAR made immunohistochemistry. DVS and DAR evaluated histopathological analysis. DVS and BPC made ELISA methods. DVS, BAR, BPC, MBV, DE, RCP, DAR, CSP interpreted the results and wrote the manuscript.

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CAPÍTULO IV

Artigo publicado na revista ***In Vivo.***

Genomic instability suppresses toll like signaling pathway in rat liver exposed to crack cocaine

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Running title: crack, toll like and genomic instability

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ABSTRACT

Background and aims. The aim of the present study was to investigate genomic damage and toll like signaling pathway in rat liver in rat liver exposed to crack cocaine.

Methods. A total of 32 rats were distributed into four groups (n = 8): Experimental group 1 (G1) and Experimental group 2 (G2): received 18 mg/kg of body weight (b.w) of crack cocaine for 5 days, once a day, the group G2 remained 72h without exposure after the experimental period (5 days)(abstinence); Experimental group 3 (G3): received 36 mg/kg of body weight (b.w) of crack cocaine for 5 days, once a day; Control Group (CTRL): received only the vehicle (DMSO) administered by intraperitoneal (i.p) route for 5 days, once a day.

Results. The groups exposed to crack had an increase in the number of micronucleated hepatocytes and binucleated cells only in the highest dose tested (36mg/kg). Karyolysis had an increase in the 18 mg/kg dose and abstinence group (G2), and 36mg/kg group (G3) whereas pyknotic nucleus had a higher average in the G2 group. The group exposed to 18mg/kg of crack cocaine also increased 8OGdG immunoexpression. The p-NF-κB p65 protein decreased in the groups exposed to crack at the doses of 18 and 36mg/kg as well as in the abstinence group. The MyD88 protein also decreased in the group exposed to crack at the dose of 18 mg/kg.

Conclusion. Taken together, our results revealed that crack cocaine inhibited toll like signaling pathway being associated with genomic instability in rat liver cells.

Key-words: crack cocaine; micronucleus; toll like signaling pathway; genomic instability; liver

1. Introduction

Crack cocaine is cocaine in the most harmful form, due to the presence of such remnants (adulterants), which are continuously used through refinement process (1). Usually, crack cocaine is presented in the form of base. The name “crack” is frequently used due to the onomatopoeic expression that refers to the crackling sound generated during pyrolysis, when the drug is consumed by smoking route (2).

Crack cocaine was first seen in Brazil in the early 90s, where it gained great popularity. Nowadays, the largest use and dissemination of crack cocaine across the country have been seen as a major public health problem, due to the severe dependence caused by using the illicit drug. As a result, risk behaviors and acts of violence are very much observed in crack cocaine users. Such behaviors affect society as a whole, putting the health and safety of the population at risk (3). There is a high prevalence of crack cocaine users visiting to the health services around the world. In the United States of America (USA), crack cocaine is responsible for the largest number of visits to health services due to the use of illicit drugs. In Brazil, the same scenario occurs, with the attendance at these services being increasingly frequent due to the indiscriminate use of crack cocaine (4).

To date, few studies have reported the toxicity induced by crack cocaine in multiple organs and tissues. Previous studies conducted by our research team have demonstrated that acute doses of crack cocaine are able to induce genetic damage in multiple organs of rodents (5,6). In humans, crack cocaine was able to increase the number of micronucleated cells in exfoliated oral mucosa cells as a result of chromosome breakage or loss (7,8).

Toll like signalling pathway plays a pivotal role in the activation of immune system being responsible for protecting the living organism. To start the toll like signalling pathway, MyD88 activates the interleukin receptor-associated kinases IRAK1 and IRAK4, which in turn stimulates the tumour necrosis-associated factor TRAF-6 adapter protein, triggering the

NFκBp50 and NFκBp65 dimers. Finally, NFκB goes to the nucleus and controls the expression of some pro-inflammatory cytokines, such as TNF-α (9). In fact, recent studies have proved that some toll like receptors mediate the inflammatory response in the hippocampus of mice exposed to cocaine (10). Others have yet reported that toll like receptor 3 plays a crucial role in cocaine addiction as a result of pro-inflammatory immune signaling in central nervous system (11). However, to the best of our knowledge, there are no studies that addressed if and to what extent, toll like signaling pathway is modulated in rat liver cells exposed to crack cocaine in vivo. This ratifies this study and others as well.

The aim of this study was to investigate if sub-acute crack cocaine exposure would be able to interfere with toll like signaling pathway associated or not with genomic instability in rat liver in vivo.

2. Material and methods

2.1 Animals and experimental design

All procedures were conducted according to the International Research Standards for Animals. The crack sample used in the present research has already undergone a chemical analysis in previous studies by the research group (5). A total of 32 Wistar male rats (*Rattus norvegicus*) weighing 250 g on average, and 9 weeks of age were distributed into four groups (n= 8), as follows: Control (CTRL); Experimental 1 (G1); Experimental 2 (G2) and Experimental 3 (G3). The experimental groups G1 and G2 received 18 mg/kg body weight (b.w.) of crack cocaine administered by intraperitoneal route (i.p.) per 5 days, once a day. The experimental group G3 received 36 mg/kg body weight (b.w.) of crack cocaine administered by intraperitoneal route (i.p.) per 5 days, once a day. Crack cocaine was also diluted in dimethylsulfoxide (DMSO). The choice of using DMSO as a vehicle was based on the view that it is a good solvent and a chelating agent, with a high diffusing capacity, easily penetrating all tissues of the organism. To standardize the amount of i.p. injection, the final volume used

was 1 mL/kg for all animals. The dose levels used of crack cocaine correspond to 25% (18 mg) and 50% (36 mg) of the cocaine lethal dose to 50% (LD50) in rodents (12). The animals of groups G1 and G3 were euthanized 5 days after i.p. injection. The animals of group G2 were euthanized 72h after the 5 days of i.p. injection.

2.2 Mutagenicity and cytotoxicity

The tissues were stained with Feulgen-Fast Green for evaluating mutagenicity and cytotoxicity. For this purpose, the following metanuclear changes were recorded: micronucleated cells, binucleated cells, pyknosis, karryorhexis and karyolyis. A total of 2000 cells were evaluated per animal.

2.3 Immunohistochemistry for 8OHdG

For imunohistochemical analysis, it was used the anti-8-hydroxy-20-deoxyguanosine (8OHdG, Santa Cruz Biotechnologies Inc.™, MO, USA) at 1:100 dilution. The method was based on increasing scores, taking into account the absence or presence of immunopositive cells associated with the extent of the stained sections, as follows: no staining (0), weak staining (1), moderate staining (2), and strong staining (3) (13).

2.4 Western blotting of Toll like signaling pathway (MyD88, TRAF-6 and NFKb-p65)

Western blotting was conducted, as described by Yujra *et al.* (14). The antibody against pNFκBp65 (sc-101744) was purchased from Santa Cruz Biotechnology™, Inc. (Santa Cruz, CA, USA). The antibodies against MyD88 (ab2064), TRAF6 (ab33915) and β-actin (ab9484) were obtained from ABCAM™ (Cambridge, UK). The intensities of each band sample were quantified by ImageJ™ software (Image J™, National Institute of Health, MD, USA). All numerical values were normalized using β-actin levels in the respective membrane.

2.5 Statistical analysis

All data were expressed as mean ± standard deviation. All analyzes were evaluated by Kruskal-Wallis non-parametric test followed by Dunn's test. $p < 0.05$ was considered to be significant.

3. RESULTS

3.1 Mutagenicity and cytotoxicity.

The groups exposed to crack had an increase in the number of micronucleated hepatocytes only in the highest dose tested (36mg/kg). Significant statistically differences ($p < 0.05$) were detected in the G3 group in relation to the control. The same occurred in the evaluation of the total number of binucleated cells, significant statistically differences ($p<0.05$) were detected in the G3 group in relation to the CTLR group. Regarding cytotoxicity, karyolysis had an increase in the dose of 18 mg/kg followed by abstinence (G2), with significant statistically differences ($p<0.05$) when compared to the CTRL group ($p\leq0.05$). The group exposed to 36 mg/kg of crack also showed an increase in karyolysis, with a significant statistically differences ($p<0.05$) when compared to the groups CTRL and G1 ($p\leq0.05$). Pyknotic hepatocytes had a higher average in the G2 group when compared to the CTRL group, with significant statistically differences ($p\leq0.05$). Karryorhexis did not show any changes among groups. Such findings are shown in **Table 1.**

Table 1. Mean ± S.D. of Metanuclar changes in rat liver induced by crack cocaine

	Metanuclear changes evaluated				
Groups *(n=08)*	**Micronucleus**	**Binucleation**	**Pyknosis**	**Karryorhexis**	**karyolysis**
CTRL	0.3 ± 0.5	92.1 ± 30.2	8.3 ± 8.3	0.7 ± 0.8	192.3 ± 71.0
G1	0.5 ± 0.7	100.5 ± 11.7	15.6 ± 8.3	0.6 ± 1.1	354.8 ± 95.5
G2	0.6 ± 0.5	72.1 ± 17.5	**22.2 ± 7.6***	0.5 ± 0.7	**421.8 ± 69.6***
G3	**1.3 ± 1.0***	**127.7 ± 12.5***	16.0 ± 6.8	1.5 ± 2.7	**563.7 ± 58.9***

Control group (CTRL); Crack 18mg/kg group (G1); Crack 18mg/kg and 72h after exposure (abstinence) group (G2); Crack 36 mg/kg group(G3). *$p\leq0.05$ when compared to CTLR.

3.2 Immunohistochemistry for 8OHdG

Immunohistochemical expression of 8OHdG was detected both in the cytoplasm and in the nucleus of liver cells. The results showed that G1, which was exposed to 18mg/kg, increased the expression of this immunomarker when compared to the CTLR group. Significant statistically differences ($p<0.05$) were detected between the groups evaluated ($p<0.05$). The abstinence group (G2) did not show any significant statistically differences ($p>0.05$) when compared to the G1 and CTLR groups ($p> 0.05$). The same occurred for the G3 group. The results are shown in **Figure 1.**

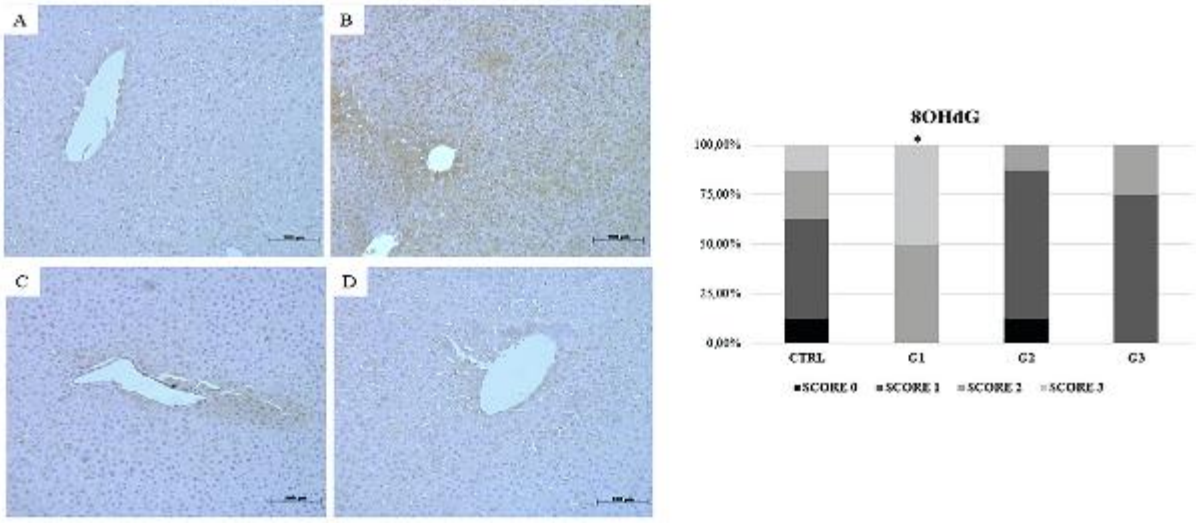

Figure 1. Photomicrographs of rat liver exposed to crack. Liver:
A) Control group (CTRL); **B)** Crack 18mg/kg group (G1); **C)** Crack 18mg/kg and 72h of wtihdrawn (G2); **D)** Crack 36 mg/kg group (G3). Photomicrography of rat liver showing cytoplasmatic staining. *$p<0.05$ when compared to CTRL group.

3.3 Protein content of Toll like signaling pathway (MyD88, TRAF-6 and NFKb-p65) by ELISA

In the hepatic tissue, the p-NF-κB p65 protein showed a decrease in the groups exposed to crack at the doses of 18 and 36mg/kg. In a similar manner, the abstinence group (G2) showed significant statistically differences ($p \leq 0.05$) in relation to the CTRL group. The MyD88 protein also showed a decrease in the groups exposed to crack at the dose of 18 mg / kg, with significant statistically differences ($p \leq 0.05$) in relation to the CTRL group. Crack cocaine was not able to modulate the expression of TRAF-6 ($p > 0.05$) in liver for all groups studied. Such findings are demonstrated in **Figure 2**.

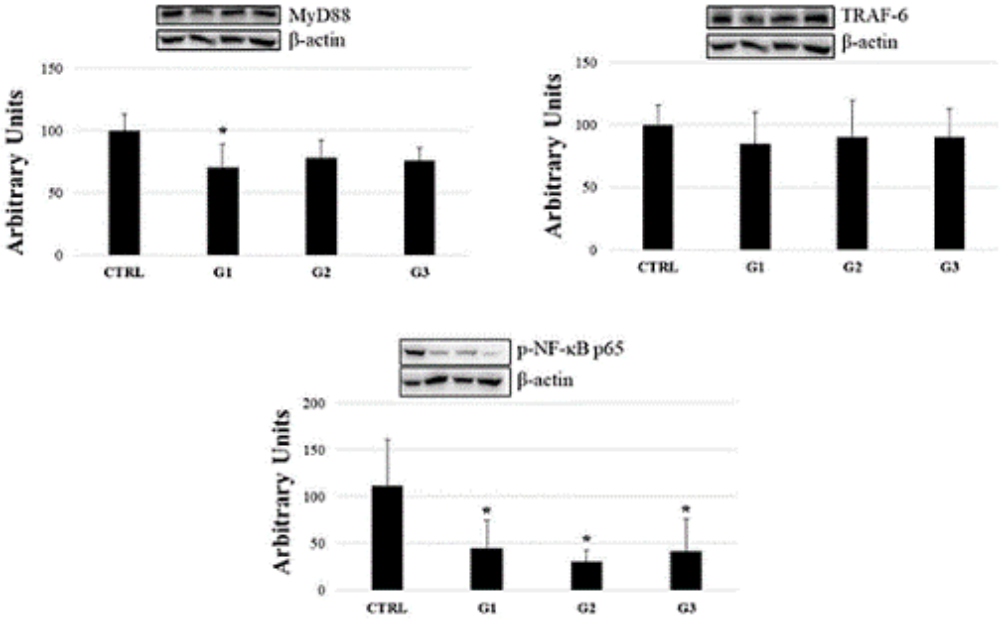

Figure 2. Toll-like signaling pathway (MyD88, TRAF-6 e p-NF-κB p65) in rat liver exposed to crack.
Control group (CTRL); Crack 18mg/kg group (G1); Crack 18mg/kg and 72h after exposure (abstinence) group (G2); Crack 36 mg/kg group(G3). *$p \leq 0.05$ when compared to CTLR.

4. DISCUSSION

The aim of this study was to evaluate cytogenetic damage, genomic oxidative stress and toll like signaling pathway in rat liver exposed to crack cocaine. First, our results demonstrated that crack cocaine at 36 mg/kg dose was able to induce mutagenicity by means of increased number of both micronucleated and binucleated cells in rat liver. Moreover, our results revealed that karyolysis was increased by crack cocaine at 36mg/kg dose. In mice, the hepatotoxic potential of cocaine was evaluated at different doses and periods of time. After 24h and 48h of drug administration, areas of necrosis in the liver parenchyma were observed. A dose of 50 mg/kg of cocaine induced hepatic tissue regeneration after 3-5 days of administration (15). Shi *et al*. (16) observed in a study with mice, necrotic areas in hepatic tissue as well. In this study, the mice received 30 mg kg of by i.p route, once a day during 3 days. Our results are fully in line with previous reports since the presence of karyolysis in rat liver has been closely related to cellular death by necrosis.

In humans, it has been demonstrated that crack cocaine increases the total number of micronucleus in buccal mucosa cells (7,8). The same results were found in human lymphocytes (17). Recently, others have assumed that crack cocaine altered the proliferative activity of buccal mucosa cells as a result of decreasing the number of AgNORs positive cells (18). However, our experimental group that remained 72h without the administration of crack cocaine after the 5 days of treatment (G2) increased the total number of pyknotic cells when compared to control group. Taken as a whole, it seems that the abstinence period after crack cocaine exposure was able to induce cellular death as a result of apoptosis. This finding is particularly important, because it is consistent with notion that damaged hepatocytes are disposed from the liver parenchyma by apoptosis after finishing the crack cocaine exposure.

8-OHdG is one of the markers used to identify oxidative damage to DNA, because among the various by-products generated during oxidative damage to DNA, changes in nitrogenous bases are synthetized in vivo and can be evaluated after DNA damage (19). The formation of 8-OHdG occurs due to the oxidation of the nitrogenous guanine base, with the addition of HO• in the 8-position carbon, giving rise to a DNA adduct, C8-hydroxy-guanine (8-OHGua). After this process, this molecule ends up losing electrons, giving rise to 8-OHdG (20). Our results demonstrated that immunoexpression for 8-OHdG increased in the group exposed to crack cocaine at 18mkg dose only. In humans, a study conducted by Bacchi *et al.* (20), found that regular cocaine users had an increase of 8-OHdG in urine. In rats, previous studies by our research group found that a single dose of 18mg/kg of crack cocaine was able to induce the formation of 8-OHdG in hepatocytes (5). The groups G2 (abstinence) and G3 (36mg/kg) did not show remarkable differences when compared to control. It is important to highlight that increased levels of 8-OHdG are correlated with the development of several pathologies, including cancer.

Regarding Toll like signaling pathway, our findings demonstrated, for the first time, that the MyD88 protein expression decreased in the group exposed to 18mg/kg of crack cocaine. With regard to the NFKB-p65 protein expression, it also decreased in all experimental groups exposed to crack cocaine (18 mg/kg and/or abstinence and 36mg/kg groups). Therefore, we assume that crack cocaine inhibited the toll like signaling pathway denoting the immunosuprressive effects in hepatocytes. Conversely, some authors have showed that cocaine increased the levels of MyD88, IRAK1, and TRAF6 in mice microglial cells (21,22). Further studies are welcomed to elucidate the issue, especially to clarity the real impact of toll like signaling proteins in different tissues and organs exposed to crack cocaine.

Taken together, our results revealed that, for the first time, that crack cocaine inhibited toll like signaling pathway being associated with genomic instability in rat liver cells.

Statement of Ethics

The study was approved by the Animal Ethics Committee of the Federal University of Sao Paulo, UNIFESP, SP, Brazil (protocol no. 7038080219).

Conflict of Interest Statement

None declared.

Funding Sources

The authors acknowledge research grants received from CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) DE Souza, ROSÁRIO thanks CAPES for grants received scholarship. RIBEIRO, PISANI and VIANA thanks CNPq for productivity fellowships.

Author Contributions

Study design: DVS, BAR, RCBS, MBV and DAR. Mutagenicity data: DVS and BAR; Immunohistochemistry data: DVS and BAR. Western blotting data: LVM and LPP. Statistics: RCBS and DAR. Writing the paper: all authors.

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CONCLUSÃO

Em suma, nosso estudo verifou que exposições subagudas de diferentes doses de crack em um modelo experimental *in vivo* foi capaz de induzir alterações no que tange a mutagenese, estresse oxidativo, inflamação, proliferação e morte celular em tecido hepático e renal de ratos *Wistar* (**Figura 13**). Verificamos efeitos citotóxicos e imunossupressores no fígado e nos rins de ratos, especialmente na dose de 36 mg / kg. No tecido hepático, observamos pela primeira vez, que o crack foi capaz de inibir a via de sinalização do Tipo Toll Like e o aumento de hepatócitos micronucleados e outras alterações metanucleares indicaram uma instabilidade genômica. Em conclusão, nossos dados oferecem novos *insights* sobre os possíveis mecanismos patobiológicos exercidos pelo crack em diversos tecidos e órgãos, uma vez que, a morte celular e a inflamação participam do processo de várias etapas da carcinogênese química.

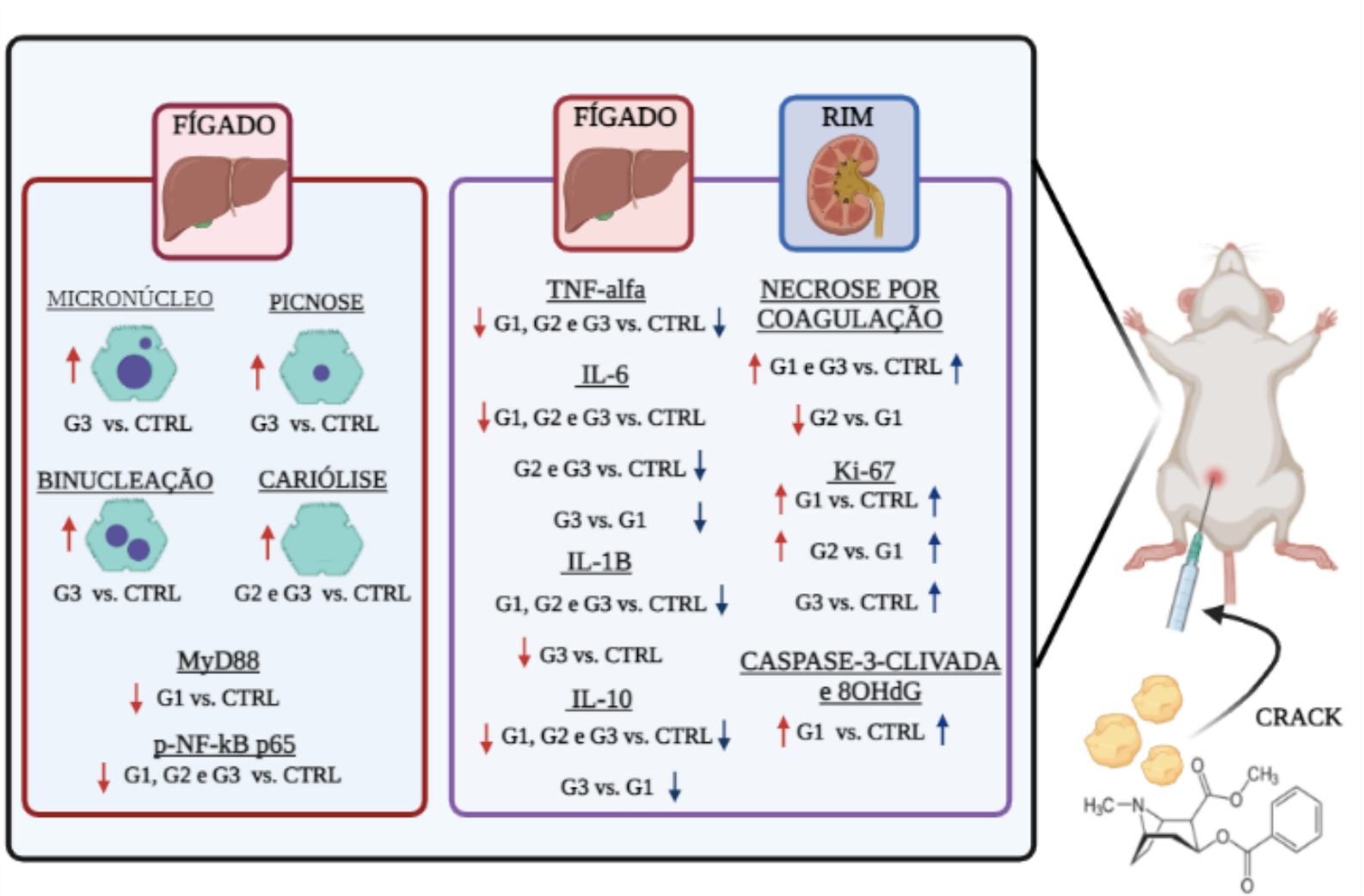

Figura 13 - Alterações no que tange a mutagenese, estresse oxidativo, inflamação, proliferação e morte celular em tecido hepático e renal de ratos *Wistar* expostos a doses subagudas de Crack.
Grupo controle (CTRL); Crack 18mg/kg (G1); Crack 18mg/kg mais período de 72h de abstinência (G2); Crack 36 mg/kg (G3). As setas para cima e para baixo aumentaram e diminuíram a frequência ou expressão, respectivamente. Setas azul: alterações no tecido renal. Seta vermelha: alterações no tecido hepático.

ANEXOS

ANEXO I

TRIBUNAL DE JUSTIÇA DO ESTADO DE SÃO PAULO
COMARCA DE LIMEIRA
FORO DE LIMEIRA
2ª VARA CRIMINAL
Rua Boa Morte, 661, ., centro - CEP 13480-181, Fone: 19 3442-5000, Limeira-SP - E-mail: limeira2cr@tjsp.jus.br

OFÍCIO

Processo nº: **3012156-28.2013.8.26.0320** **Ordem nº 1122/2013**
Classe – Assunto:
Representante: **UNIVERSIDADE FEDERAL DE SÃO PAULO (UNIFESP) - CAMPUS BAIXADA SANTISTA**
(FAVOR MENCIONAR ESTAS REFERÊNCIAS NA RESPOSTA)

O(A) MM. Juiz(a) de Direito da 2ª Vara Criminal do Foro de Limeira, Dr(a). Luiz Augusto Barrichello Neto, pelo presente, expedido nos autos da ação em epígrafe, comunica à Vossa Senhoria que foi deferido o pedido para liberação de 50 gramas de crack para realização de pesquisa científica, ficando consignado que o transporte da substância entorpecente ficará ao encargo da Universidade Federal de São Paulo, através de pessoa responsável que deverá apresentar-se documentalmente à Delegacia de Investigações sobre Entorpecentes de Limeira. Após a realização dos estudos, a substância deverá ser totalmente inutilizada, com acompanhamento da Vigilância Sanitária, encaminhando-se cópia do relatório à este Juízo, tudo conforme cópias integrais dos autos que seguem em anexo, referentes a parte abaixo qualificado(a)(s):
Representante: UNIVERSIDADE FEDERAL DE SÃO PAULO (UNIFESP) - CAMPUS BAIXADA SANTISTA, SILVA JARDIM, 136, Santos-SP, Brasileiro

Atenciosamente.

Limeira, 18 de outubro de 2013.

DOCUMENTO ASSINADO DIGITALMENTE NOS TERMOS DA LEI 11.419/2006, CONFORME IMPRESSÃO À MARGEM DIREITA

Ao Ilmo Sr. Reitor da
UNIVERSIDADE FEDERAL DE SÃO PAULO
Rua Silva Jardim, 136 - Santos
SANTOS/SP – CEP 11015-020

Prof. Daniel Araki Ribeiro

ANEXO II

UNIFESP
UNIVERSIDADE FEDERAL DE SÃO PAULO

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "ATIVIDADE INFLAMATÓRIA, OXIDANTE E PROLIFERATIVA EM FÍGADO E RIM DE RATOS EXPOSTOS AO CRACK", protocolada sob o CEUA nº 7038080219 (ID 008257), sob a responsabilidade de **Daniel Vitor de Souza** *e equipe; Daniel Araki Ribeiro; Carolina Foot Gomes de Moura* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de São Paulo (CEUA/UNIFESP) na reunião de 13/03/2019.

We certify that the proposal "INFLAMATORY, OXIDANT AND PROLIFERATIVE ACTIVITY IN LIVER AND KIDNEY OF RATS EXPOSED TO CRACK COCAINE", utilizing 24 Heterogenics rats (24 males), protocol number CEUA 7038080219 (ID 008257), under the responsibility of **Daniel Vitor de Souza** *and team; Daniel Araki Ribeiro; Carolina Foot Gomes de Moura* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Sao Paulo (CEUA/UNIFESP) in the meeting of 03/13/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 06/2019 a 12/2020 Área: Patologia

Origem: Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia - CEDEME

Espécie: Ratos heterogênicos sexo: Machos idade: 9 a 9 semanas

N: 24 Linhagem: Wistar Peso: 250 a 250 g

Local do experimento: Laboratório de Toxicogenômica - UNIFESP - Campus Baixada Santista

São Paulo, 11 de junho de 2019.

Profa. Dra. Monica Levy Andersen
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de São Paulo

Profa. Dra. Daniela Santoro Rosa
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de São Paulo

ANEXO III

ARTIGOS PUBLICADOS DURANTE O MESTRADO

ARTIGO 1: Publicado na Revista Asian Pacific Journal of Cancer Prevention.

GENOMIC INSTABILITY IN PERIPHERAL BLOOD AND BUCCAL MUCOSAL CELLS OF MARIJUANA SMOKERS: THE IMPACT OF TOBACCO SMOKE

Daniel Vitor De Souza[1] , Samuel Rangel Claudio[1] , Camila Lima Feitosa Da Silva[2] , Kevin Pereira Marangoni[2], Rogerio Correa Peres[2], Daniel Araki Ribeiro[1]*

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DOI:10.31557/APJCP.2020.21.5.1235
Genetic Damage in Marijuana Users

RESEARCH ARTICLE

Editorial Process: Submission:02/26/2019 Acceptance:13/10/2019

Genomic Instability in Peripheral Blood and Buccal Mucosal Cells of Marijuana Smokers: The Impact of Tobacco Smoke

Daniel Vitor De Souza[1], Samuel Rangel Claudio[1], Camila Lima Feitosa Da Silva[2], Kevin Pereira Marangoni[2], Rogerio Correa Peres[2], Daniel Araki Ribeiro[1]*

Abstract

Background: The aim of this study was to evaluate cytotoxic, mutagenic and genotoxic effects on buccal mucosa and peripheral blood cells from marijuana and tobacco smokers. **Methods:** For this purpose, a total of 45 volunteers were distributed into four groups: CTRL group (control): individuals who did not smoke marijuana or tobacco (n = 11); Group M: Marijuana smokers (n = 13); Group T: Tobacco smokers (n = 13); Group M + T: Smokers of both marijuana and tobacco (n = 08). **Results:** Smokers of both marijuana and tobacco led an increase of micronucleated cells on buccal mucosa when compared to control group. The occurrence of karyolysis showed significant changes in this group as well. The comet assay data revealed genetic damage in peripheral blood cells for all groups of smokers. **Conclusion:** In summary, our results showed that marijuana and /or tobacco are able to induce genetic damage and cytotoxicity in oral and peripheral blood cells.

Keywords: Oral mucosa cells- peripheral blood- marijuana- tobacco smoke- micronucleus test

Asian Pac J Cancer Prev, **21 (5)**, 1235-1239

Introduction

The plant Cannabis sativa belongs to the family Cannabaceae L, popularly known as marijuana from Asian continent (Lopez et al., 2014). The harvest occurs in the fall to have a good concentration of its main bioactive compound, Delta-9-tetrahydrocannabinol (THC) (Lopez et al., 2014). To date, there are several variables for consuming the plant, such as inhalation (smoking), ingestion (use of the plant in homemade recipes, such as cakes and biscuits) and skin absorption (adhesives). It is important to stress that the consumption method chosen directly affects the onset of its effect as well as the amount of THC that will be absorbed by the organism. The inhalation promoted by cigarettes in dry form is widely used due to greater effectiveness. It is estimated that 0.5-1 g of the plant contains 20 mg of THC. This in turn is consumed in the form of tetrahydrocannabinolic acid as a result of combustion of the plant (Bonfa et al., 2003). The acid then converts into free THC, where it is absorbed through inhalation; the smoke goes to the lungs allowing the bioactive substances to reach the bloodstream and central nervous system (Bonfa et al., 2003).

Marijuana is considered an illicit drug in many countries, such as Brazil, where it is widely consumed by people in general who justify its use to produce relaxation, and decreased stress and anxiety (Zuardi et al., 2010). Nevertheless, it has been documented that marijuana smoke promotes several harmful health effects (Lee and Hancox, 2011). In fact, some authors have postulated that marijuana smoke condensates contain similar chemical compounds as those detected in tobacco cigarette smoke (Moir et al., 2008). Many of them, have been classified as carcinogens by International Agency for Research on Cancer (IARC) (Smith et al., 2003). Herein, it would be important to know whether, and to what extent, marijuana smoke condensates, associated or not with tobacco cigarette smoke, could exert harmful effects on human health, especially on genetic material. This investigation is relevant since there are few studies investigating marijuana-only smoking populations as well as the association with tobacco cigarette smoke.

To date, some research groups have investigated putative biomarkers for biomonitoring continuous exposure of human populations to environmental mutagens and carcinogens (Maranho et al., 2017; Claudio et al., 2019). Among them, micronucleus and single cell gel comet assays are simple, low cost, reproducible and non-invasive methodologies for investigating cytogenetic damage and DNA strand breaks in eukaryotic cells, respectively (Tice et al., 2000; Bonassi et al., 2011). Previous studies conducted by our research group have demonstrated that micronucleus and single cell gel comet assays are important tools for biomonitoring people

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ARTIGO 2: Publicado na Revista Journal of Endocrinology.

HEPATIC INFLAMMATION PRECEDES STEATOSIS AND IS MEDIATED BY VISCERAL FAT ACCUMULATION

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Journal of Endocrinology | B P Casagrande et al. | Hepatic inflammation precedes steatosis | 245:3 | 369–380

RESEARCH

Hepatic inflammation precedes steatosis and is mediated by visceral fat accumulation

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Abstract

The negative aspects of unhealthy eating on obesity and hepatic health are well described. The axis between the adipose tissue and the liver participates in most of the damage caused to this tissue regarding obesogenic diets (OD). At the same time that the effects of consuming simple carbohydrates and saturated fatty acids are known, the effects of the cessation of its intake are scarce. Withdrawing from OD is thought to improve health; despite some studies had shown improvement in hepatic conditions in the long-term, short-term studies were not found. Therefore, we aimed to determine how OD intake and withdrawal would influence visceral and hepatic fat accumulation and inflammation. To this end, male 60-days-old Wistar rats received standard chow (n = 16) or a high-sugar/high-fat diet (HSHF) for 30 days (n = 32), a cohort of the HSHF-fed animals was then kept 48 h on standard chow (n = 16). In opposition to the generally reported, the results indicate that hepatic inflammation preceded hepatic steatosis. Additionally, inflammatory markers on the liver positively correlated visceral adipokines and visceral fat accumulation mediated them in a deposit-dependent manner. At the same time, a 48-h withdrawal was capable of reverting most of the risen inflammatory mediators, although MyD88 and TNFα persisted and serum non-HDL cholesterol was higher than control levels.

Key Words

- hepatic inflammation
- hepatic steatosis
- visceral adipose tissue
- obesogenic diet
- withdrawal

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Introduction

The negative aspects of obesogenic diets (OD) on adipose tissue accumulation, hepatic fat accumulation (steatosis), and inflammation are well described (Van Herck *et al.* 2017, Bortolin *et al.* 2018, Casagrande *et al.* 2019). Obesity and non-alcoholic fatty liver disease (NAFLD) are two parts of the same role, respectively, as causation and consequence of the metabolic syndrome (Kanwar & Kowdley 2016). It is believed that following the adipocyte-hepatocyte axis, an increase in adipose tissue deposits drives an increase in the hepatic fat content, mainly as triacylglycerol (TAG) (Smith & George 2009). NAFLD can progress to non-alcoholic steatohepatitis (NASH), with higher inflammation (Kanwar & Kowdley 2016).

The adipocyte-hepatocyte axis can promote an over-activation of the toll-like receptors pathway and the production of inflammatory cytokines. Likewise, simple carbohydrates and fatty acids from the diet produce this effect (Wagnerberger *et al.* 2012, Rocha *et al.* 2016, Totsch *et al.* 2017). This pathway can be mediated by myeloid differentiation factor 88 (MyD88) and lead to the phosphorylation and nuclear translocation of the nuclear factor kappa B (NFκB) culminating in cytokine production (Lu *et al.* 2008).

The harms caused by unhealthy eating are widely known (Morris *et al.* 2015). Meanwhile, despite improving eating habits being recommended for populations

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ARTIGO 3: Publicado na Revista World Journal of Advanced Research and Reviews.

BACTERIAL CELLULOSE MEMBRANE ENRICHED WITH FIBROBLAST GROWTH FACTOR ASSOCIATED WITH PHOTOBIOMODULATION: IN VITRO EVALUATION

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[RESEARCH ARTICLE]

Bacterial cellulose membrane enriched with fibroblast growth factor associated with photobiomodulation: *In vitro* evaluation

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Abstract

One promising skin substitutes in the wound healing are the bacterial cellulose membranes (BCM). These biomaterials present nanostructures composed of microfibrils capable of forming three-dimensional pores that allow cell. In association with these biopolymers, several treatments are used, such as enrichment by growth factors and/or the application of photobiomodulation (PBM). Therefore, the aim of this study was to investigate the viability, proliferation and cytotoxicity of a BCM (culturing of Komagataeibacter xylinus), with or without FGF-2 in association with PBM therapy. In the characterization of BCM we saw that the membrane does not show great variations in pH and with the scanning electron microscopy it was possible to observe that the BCM has a denser and a porous side that allows the adhesion of fibroblasts, confirmed by histological staining and DAPI/Phalloidin. In vitro evaluation showed that the immunofluorescence (CaAM/EthD-1) for live and dead cells presented, in the groups with combined treatment at long-term of PBM and FGF-2, a greater quantity of live cells than with these isolated treatments and/or at short-term. However, in the short-term of combined treatment PBM and FGF-2 supplementation, fibroblasts and macrophages were more viable by Alamar Blue, in direct and indirect contact respectively. The comet assay did not show cytotoxicity for DNA damage in fibroblasts indirect contact with membrane extract. The results highlight the potential of association of FGF-2 supplementation with the application of PBM for use with BCM, due to its promoted increased cell density at long-term and improved viability in fibroblasts and macrophages at short-term.

Keywords: Bacterial cellulose membrane; Biopolymers; Fibroblast growth factor; Photobiomodulation; Fibroblast; Macrophages.

1. Introduction

In the area of tissue engineering there are great efforts to improve the tissue regeneration process of skin injuries in terms of the development of biomaterials that serve as a substitute for injured skin [1]. Skin substitutes can be classified into epidermal, dermal and epidermal-dermal and trilayered, replacing the dermal layers according to their names.

The most commonly used types of substitutes are gels, creams, resins, oils, films, membranes, scaffolds and grafts [2,3]. These biomaterials can be made using several materials such as: collagen, fibrin, alginate, hyaluronic acid and bacterial cellulose [4].

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ARTIGO 4: Publicado na Revista European Journal of Radiology.

THE IMPORTANCE OF GENOTOXICITY STUDIES FOR BIOMONITORING CHILDREN EXPOSED TO X-RAY

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Correspondence

The importance of genotoxicity studies for biomonitoring children exposed to X-ray

ARTICLE INFO

Keywords:
Micronucleus assay
Buccal cells
X-ray
Children

We read the manuscript recently published by Milić et al. [1] in the European Journal of Radiology titled "Application of the buccal micronucleus cytome assay on child population exposed to sinus X ray" with much interest. In this article, the authors failed to detect high frequencies of micronucleus followed by significant increase of condensed chromatin in buccal cells of children submitted to sinus X-ray. However, the manuscript presents some technical issues that are important to discuss for a correct understanding the scientific approach.

In Material and Methods, it was stated "The buccal MN cyt assay was performed according to Thomas et al. with minor modifications". Since minor modifications were performed, the authors must state what stain was used in the study. This information is very important, because the micronucleus assay should only be performed by stains that are specific for nucleic acids. Otherwise, this inevitably leads to false positive results due to the identification of cell structures that resembling micronucleus, such as keratohyalin granules or bacteria [2]. Furthermore, it was written that "A minimum of 1000 cells were counted to determine the frequency of each cell type in the sample" and then "DNA damage biochildren. Some authors have postulated that condensed chromatin is closely associated with the apoptosis process (a programmed mechanism of cellular death) [4]. Nevertheless, it is important to stress that cytotoxicity is a confounding factor for genotoxicity [5]. If cytotoxicity is increased, the micronucleus frequency automatically decreases, because micronucleated cells are lost due to cellular death. This needs be discussed in the manuscript.

We hope that such comments are useful for the correct understanding of the paper biomonitoring children submitted to X-ray.

Author contributions

All authors wrote the Letter to the Editor.

Declaration of Competing Interest

The authors report no declarations of interest.

ARTIGO 5: Publicado na Revista Asian Pacific Journal of Cancer Prevention.

CYTOGENETIC BIOMONITORING ON ORAL MUCOSA CELLS OF HOOKAH USERS: IS IT POSSIBLE?

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Cytogenetic Damage on Hookah Users

LETTER to the EDITOR

Editorial Process: Submission:04/30/2019 Acceptance:07/13/2020

Cytogenetic Biomonitoring on Oral Mucosa Cells of Hookah Users: Is It Possible?

Asian Pac J Cancer Prev, **21 (7), 1849-1849**

Dear Editor

Genotoxicity and cytotoxicity are defined as the ability of an agent to cause injury on genetic material and to induce cellular death, respectively. These occurrences have clinical implications because DNA damage and cell death are closely involved to several diseases including cancer. Herein, this is important to understand what chemical agents induce harmful effects on oral cells under different contexts and paradigms. This information will contribute to a better understanding the pathways closely involved to genotoxicity/cytotoxicity in order to prevent diseases in the oral cavity. The manuscript recently published by Taghibakhsh et al., (2019) have assumed that Hookah use is able to induce chromosome breakage and cellular death in oral mucosa cells by means of micronucleus test. However, this study has some issues that motivates us to establish a discussion about the matter.

In the hookah group (10.7) compared to the control group (5.8) (P<0.001), The mean percentages of KR in the control and case groups were 0.04 and 0.1, respectively, which was 2.5 folds higher in the case group compared to the control group (P<0.001). In addition, the mean percentages of KL in the control and case groups. were 0.08 and 0.16, respectively, which was 2 folds higher in the control group compared to the case group (P<0.026). The repair index in the control and test groups were 0.05 and 0.03, respectively (Table 2), which was 40% higher in the control group compared to the case group (P<0.026)". Unfortunately, the results presented in Tables 1 and 2 are inverted as those described by the authors. This requires further clarification.

We believe that these comments be useful for better understanding the important article investigating cytogenetic damage on oral mucosa cells of Hookah users.

ARTIGO 6: Publicado na Revista Asian Pacific Journal of Cancer Prevention.

IS ORTHODONTIC THERAPY ABLE TO INDUCE GENETIC DAMAGE ON ORAL CELLS?

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LETTERS TO THE EDITOR

WILEY Orthodontics & Craniofacial Research

Is orthodontic therapy able to induce genetic damage on oral cells?

We read the manuscript of Faccioni et al[1] recently accepted for publication in the Orthodontics & Craniofacial Research titled "Short-term 'In vivo' study on cellular DNA damage induced by acrylic Andresen activator in oral mucosa cells" with much interest. In this article, the authors found DNA breakage and high frequencies of micronuclei on buccal cells from orthodontically treated patients when compared to controls. However, this study has some issues that must be properly clarified for better understanding the paper.

In this study, the authors applied trypan blue exclusion test for evaluating cytotoxicity. It is important to mention that the principle of this methodology is the cell membrane integrity rather than cellular activity or nuclear changes able to predict cell death. For this reason, the sensitivity of the assay is very low. Probably, this may explain the negative result found by the authors. Tolbert et al[2] purposed to evaluate metanuclear changes indicative of cytotoxicity when analysing micronucleus assay in buccal mucosa cells, such as pyknosis, karyolysis and karyorrhexis. The approach could be included in this study for improving cytotoxicity data. Another the significant increase of TI rather than TL. This datum could indicate that adducts formation could be another important mechanism by which the resin monomers cause DNA damage, in addition to ROS production already observed in vitro.[3] Nevertheless, the results showed that TM and TI increased following exposure to acrylic Andresen activator, but not TL. Moreover, the single cell gel (comet) assay detects a wide range of DNA lesions including adducts, single- and double strand breaks, alkali labile sites and incomplete repair sites.[4] Taken together, we assumed that such DNA lesions should be considered when interpreting the comet assay data. In the 3rd paragraph, the authors state that "Comet assay revealed that resin monomers released by AA initially penetrated the most superficial buccal mucosa epithelial cells inducing DNA damage, subsequently arriving in the germinative layer, they could induce DNA breaks or interfere with the mitotic spindle." The statement does not make sense since comet assay is not able to evaluate genetic damage in the germinative layer. Micronucleus assay is suitable for this purpose.

ARTIGO 7: Publicado na Revista Iranian Journal of Pathology.

IS MICRONUCLEUS ASSAY SUITABLE FOR CYTOGENETIC BIOMONITORING THE DIFFERENT WAYS TO SMOKE?

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Is Micronucleus Assay Suitable for Cytogenetic Biomonitoring the Different Ways to Smoke?

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Dear Editor,

We read the manuscript of DehghanNezhad *et al.* (1) recently published in the Iranian Journal of Pathology titled "Micronucleus Assay of Buccal Mucosa Cells in Waterpipe (Hookah) Smokers: A Cytologic Study." with much interest. In this article, the authors were able to detect high frequencies of micronucleus in buccal mucosa cells of waterpipe smokers when compared to non-smokers. However, it is important to properly assess the scientific approach for a correct understanding of the paper.

First, it is important to stress that some criteria were established for the correct identification of micronucleus in buccal mucosa cells. In Material and Methods part, the authors stated that "The structures within cytoplasm with similar staining of nucleus measuring between 1/5 to1/3 size of nucleus was considered as micronucleus".

We believe that these comments can be useful for better understanding the relevant study on cytogenetic biomonitoring on buccal mucosa cells of waterpipe smokers.

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Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

ARTIGO 8: Publicado na Revista Environmental Science and Pollution Research.

COMMENTS ON "MONITORING HUMAN GENOTOXICITY RISK ASSOCIATED TO URBAN AND INDUSTRIAL BUENOS AIRES AIR POLLUTION EXPOSURE" BY LOS A GUTIÉRREZ ET AL. (2020)

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LETTER TO THE EDITOR

Comments on "Monitoring human genotoxicity risk associated to urban and industrial Buenos Aires air pollution exposure" by Los A Gutiérrez et al. (2020)

Daniel Vitor de Souza[1] · Andrea Cristina de Moraes Malinverni[1] · Daniel Araki Ribeiro[1]

Received: 7 March 2020 / Accepted: 25 August 2020

We read the recent paper accepted for publication in *Environmental Science and Pollution Research* titled "Monitoring human genotoxicity risk associated to urban and industrial Buenos Aires air pollution exposure" by de Los A Gutiérrez et al. (2020) with much interest. However, this study has some questions that must be clarified for better understanding the manuscript with accuracy.

In Material and Methods, Study Population section, it was written that "Exclusion criteria included pregnancy or breastfeeding (in female volunteers), drug addictions, and smoking." However, in the Results it was stated that "In order to validate our experimental protocol, we first analyzed

It is important to stress that the authors write "nuclear aberrations assay" throughout the manuscript. In our opinion, this is not correct because some of metanuclear changes evaluated are physiologically found during turnover of the oral mucosa as, for example, pyknosis, karyorrhexis, and karyolysis. The term correct is "micronucleus assay."

In Results, it was stated that "we evaluated nuclear aberrations within three age intervals: young adult (18-35), middle-aged adult (36-55), and older adult (>55) (data not shown)." Again, what is the total number of participants (n) in each subgroup? Anyway, these data should be included in the manuscript because cytotoxicity is a confounding factor when in-