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Dannyele Cynthia Santos Pimentel Nicácio

ALTERAÇÕES MORFOFUNCIONAIS SALIVARES E ÓSSEAS EM RATOS JOVENS EXPOSTOS À FUMAÇA DO *CRACK* NO PERÍODO INTRAUTERINO

Maceió, 2019

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Dissertação apresentada ao Programa de Pós-graduação em Ciências da Saúde da Universidade Federal de Alagoas – Instituto de Ciências Biológicas e da Saúde, como requisito para a obtenção do Título de Mestre em Ciências da Saúde.

Orientador: Prof. Dr. Marcelo Duzzioni
Coorientador: Prof. Dr. Robinson Sabino da Silva

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DANNYELE CYNTHIA SANTOS PIMENTEL NICÁCIO

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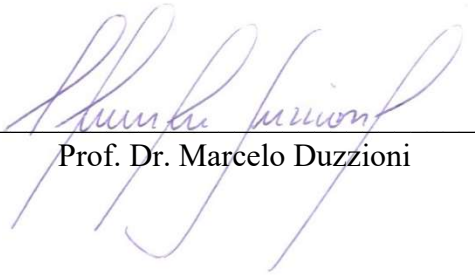
Relatório final apresentado a Universidade Federal de Alagoas, como parte as exigências para título de Mestre em Ciências da Saúde.

Maceió, 29 de março de 2019

BANCA EXAMINADORA



Prof. Dr. Marcelo Duzzioni

Prof. Dr. Olagide Castro

Dedicatória

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Epígrafe

" Consagre ao Senhor tudo o que você faz, e os seus planos serão bem-sucedidos" (Pv. 16:3)

Resumo

O uso de substâncias aditivas durante o período gestacional é um importante problema social e de saúde pública. Nos últimos anos tem chamado a atenção o uso indiscriminado do *crack* (cocaína na forma base livre) por mulheres grávidas. O *crack* tem efeitos deletérios à mãe e ao recém-nascido, incluindo anomalias cardíacas, baixo peso ao nascer, menor comprimento ósseo e menor perímetro encefálico. O objetivo do presente estudo foi avaliar os efeitos no fluxo e na composição salivar, bem como a composição e a microarquitetura do tecido ósseo (fêmur) de animais expostos à fumaça do *crack* durante o período gestacional. Para isso, ratas Wistar prenhes foram expostas durante o 5º e o 21º dia do período gestacional à fumaça de *crack* por 5 min. Os animais do grupo controle permaneceram nas caixas-moradia durante o período de exposição. Passados 30 dias do nascimento, ratos machos do grupo exposto à fumaça do *crack* e controle foram anestesiados e divididos em dois grupos. Após a confirmação da anestesia, os animais do primeiro grupo foram tratados com o agente sialogênico pilocarpina (agonista muscarínico não seletivo, 2 mg/Kg, i.p.) e, em seguida, a secreção salivar foi coletada por 10 min. Ao final, os animais foram eutanasiados e as glândulas salivares (parótida, submandibular e sublingual) foram coletadas e pesadas. Através da técnica de espectroscopia no infravermelho com transformada de Fourier (FTIR) foi analisada a composição da saliva. O outro grupo de animais expostos e controle foram eutanasiados para a retirada do fêmur direito e posterior análise do perfil químico e da microarquitetura ósseo através das técnicas FTIR e microtomografia computadorizada (Micro-CT), respectivamente. Nossos resultados não demonstraram diferenças significativas no fluxo e no peso das glândulas salivares, bem como no conteúdo de amida, bases nitrogenadas, amida I, proteínas e frações de açúcar entre os grupos experimentais ($p > 0,05$). Em relação aos ensaios ósseos, nossos resultados demonstraram que a exposição à fumaça do *crack* não alterou de forma significativa o conteúdo de grupos ésteres de ácidos graxos, amida I e II, curvaturas de proteínas por metileno, fosfato I, colágeno e carbonato, quando comparado ao grupo controle. Entretanto, a conformação simétrica CH3 foi aumentada no grupo *crack*. No ensaio de Micro-CT, não houve diferença significativa entre os grupos experimentais para os parâmetros ósseos trabeculares ($p > 0,05$). Por outro lado, os parâmetros do osso cortical foram aumentados no grupo *crack*, que obteve uma maior dimensão fractal ($p < 0,05$) e maior número de poros fechados ($p < 0,01$), comparado ao grupo controle. Análise estatistica foi realizada através do teste t de Student não pareado e teste não paramétrico de Mann-Whitney; valores de $p < 0,05$ foram considerados estatisticamente significantes. Dessa forma, nossos resultados indicam que à exposição à fumaça do *crack* durante o período gestacional não altera a fluxo e a composição salivar das proles um mês após o nascimento. Entretanto, à exposição à fumaça do *crack* durante o período gestacional aumentou os parâmetros de qualidade óssea, possivelmente como um fator de proteção contra os metabólitos do *crack*.

Palavras-chaves: *Crack* gestacional, Osso, Saliva , FTIR, Micto-CT.

Abstract

The use of additive substances during the gestational period is an important social and public health problem. In recent years attention has been drawn to the indiscriminate use of crack cocaine by pregnant women. Crack cocaine has deleterious effects on the mother and newborn, including cardiac abnormalities, low birth weight, shorter bone length and lower brain perimeter. The objective of the present study was to evaluate the effects on salivary flow rate and composition, as well as, the composition and microarchitecture of the bone tissue (femur) of animals exposed to crack cocaine during the gestational period. For this, pregnant Wistar rats were exposed during the 5th and 21st days of the gestational period to crack cocaine for 5 min. Control animals remained in the housing boxes during the exposure period. Postnatal day 30, male rats exposed to crack cocaine during the intrauterine period and control were anesthetized and divided into two groups. After confirmation of the anesthesia, the animals were treated with the sialogenic agent pilocarpine (non-selective muscarinic agonist, 2 mg/kg, i.p.) and then the salivary secretion was collected for 10 min. At the end, the animals were euthanized and the salivary glands (parotid, submandibular and sublingual) were collected and weighed. The composition of the saliva was analyzed by Fourier transform infrared spectroscopy (FTIR). The other group of exposed and control animals were sacrificed for the removal of the right femur and subsequent analysis of the chemical profile and bone microarchitecture through FTIR and micro-computed tomography (Micro-CT), respectively. Our results did not show significant differences in the flow rate and weight of the salivary glands, as well as in the amide, nitrogenous bases, amide I, proteins and sugar fractions between the experimental groups ($p > 0.05$). In relation to the bone assays, our results demonstrated that exposure to crack cocaine did not significantly alter the content of fatty acid esters, amide I and II groups, protein curves by methylene, phosphate I, collagen and carbonate when compared to the control group. However, the CH3 symmetrical conformation was increased in the crack group. In the Micro-CT assay, there was no significant difference between the experimental groups for the trabecular bone parameters ($p > 0.05$). On the other hand, cortical bone parameters were increased in the crack group, which obtained a larger fractal dimension ($p < 0.05$) and higher number of closed pores ($P < 0.01$), purchased from the control group. Statistical analysis was performed through unpaired Student t-test and non-parametric Mann-Whitney test; values of $p < 0.05$ were considered statistically significant. Thus, our results indicate that exposure to crack cocaine during the gestational period does not alter the salivary flow rate and composition of offspring until one month after birth. However, exposure to crack cocaine during the gestational period increased bone quality parameters, possibly as a protective factor against crack metabolites.

Key-words: Gestational Crack cocaine, Bone, Saliva, FTIR, Micto-CT.

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Capítulo 1

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Lista de Abreviaturas e Siglas

µl – microlitro

µm - micrometro

3D – three dimensions

a.m. – after meridiem

a.u. – astronomical unit

AEME - methyl ester anhydroecgonine

BIOCEN – biotério central

BV – bone volume fraction

CEUA – comitê de ética de uso de animais

cm^{-1}- número de onda em centimetros

DA – degree of anisotropy

FD – fractal dimension

FTIR - Fourier transform infrared spectroscopy

i.p. - intraperitoneal

J – joule

Kg - kilograma

mg - miligrama

Micro-CT - computerized microtomography

min - minuto

ml - mililitro

mm - milimetro

n - número

p.m. – post meridiem

PoN(cl) – number of closed pores

PPE - personal protective equipment

s- segundo

Tb.sp – trabeular separation

TbN – trabecular number

TbTh – trabecular thickness

TV – tissue volume fraction

UFAL – Universidade Federal de Alagoas

Sumário

1. Introdução e Referencial Teórico

1.1. Crack: definição, epidemiologia e seu uso durante a gestação

O *crack* é a forma de base livre da cocaína (FREITAS *et al*., 2014).

A cocaína é um alcaloide extraído das folhas da *Erythroxylum coca*, uma planta originária dos altiplanos andinos, que produz múltiplas ações farmacológicas periféricas e centrais. A cocaína produz seus efeitos psicoativos e aditivos principalmente por agir no sistema límbico, um conjunto de regiões cerebrais interconectadas que regulam prazer e motivação (Nestler, 2005). Esses efeitos são em parte causados pela inibição da recaptação de dopamina nas vias mesolímbica e mesocortical. E o uso frequente da cocaína promove uma diminuição no conteúdo de dopamina nessas áreas cerebrais, havendo, portanto, a necessidade de mais consumo da droga para conseguir o efeito desejado (MATOS *et al.,* 2011). Além de afetar o sistema domaninérgico, há também a inibição de receptação da noradrenalina e serotonina, podendo causar efeitos como: hipertensão e alterações do sono, respectivamente (MATOS *et al.,* 2011). A cocaína pode ser administrada por via endovenosa ou intranasal (PARCIANELLO *et al.,* 2018; FREITAS *et al.,* 2014). A forma inalada (fumada) é através do seu subtipo o *crack,* que pode ser originado da pasta base de cocaína, ou ainda da coicaína proprimante dita, sua forma mais purificada, entretanto, com adição de produtos inertes para baratear sua produção (FISCHMAN, 1988).

O termo *crack* surgiu nos Estados Unidos, na década de 80, devido aos ruídos típicos de estalos que a droga faz quando aquecida (MATOS *et al.,* 2011). O *crack* possui efeitos mais deletérios que a cocaína usada de forma intranasal ou injetável. A via de administração está interligada com o risco dependência dessa droga, enquanto a cocaína intranasal demora de 2 a 3 minutos para chegar ao sistema nervoso central, o *crack* leva de 8 a 10 segundos. Além disso, o efeito do *crack* dura cerca de 10 minutos, sendo mais rápido e intenso, bem como os sinais de dependência, quando comparado ao da cocaína intranasal, que dura de 30 a 45 minutos (FISCHMAN, 1988). O *crack*, devido aos problemas com criminalidade e outras deficiências sociais, apresenta pior prognóstico quando comprado a outras drogas (MORETTI *et al.,* 2016).

A dependência ao *crack* é um problema de saúde pública mundial. O *crack* foi introduzido no Brasil no final dos anos 1980 e início dos 90, aumentando ao decorrer das décadas em todas as classes sociais (MORETTI *et al.,* 2016). Em 2016, a Secretaria

Nacional de Política sobre Drogas, do Ministério da Justiça e Cidadania, publicou um livro, revelando que o Brasil possui cerca de 370 mil usuários regulares de *crack* nas capitais. Em 2013, a Fundação Oswaldo Cruz em associação com o Ministério da Saúde e da Justiça relatou que 35% do total de consumidores de drogas ilícitas eram usuários de *crack*, sendo que 50% das mulheres entrevistadas relataram que já haviam engravidado ao menos uma vez desde que iniciaram o uso do *crack*, sendo que 10% destas estavam grávidas no momento da pesquisa. Destaca-se ainda que para as usuárias de *crack*, a gravidez nunca foi um empecilho para o consumo da droga, conforme Yandow (2011). Além disso, as gestantes usuárias de *crack* negligenciam o acompanhamento pré-natal (MATOS *et al.,* 2011). Essa dependência do *crack* durante a gestação, que provavelmente irá continuar após o nascimento, dificulta a interação materno-infantil, já no período gestacional, e agrava-se após a manifestação dos efeitos teratogênicos causados pela exposição ao *crack*.

1.2. *Crack* X Saliva X Osso

Um dos efeitos adrenérgicos devido a inbição da receptação da noradrenalina é a hipossalivação (MATOS *et al.,* 2011). A ocorrência desse efeito é bem relatada na literatura em usuários de *crack*/cocaína (ANTONIAZZI *et al.*, 2017; SORDI *et al*., 2017; WOYCEICHOSKI *et al.,* 2013). Etretanto não há estudos que associem esse efeito em fetos que foram expostos a fumaça do *crack* no período intratuterino.

Na estrutura ósseoa não há relatos de comprometimento ou alterações em usuários de *crcak*/cocaína, em contrapartida é observado um menor comprimento ósseo quando ocorre essa exposição pré-natal à fumaça do *crack* (CAMPILLO *et al.,* 2004).

1.3. Saliva

A saliva é um fluido biológico formado por secreções produzidas pelas glândulas salivares maiores (parótida, submandibular e sublingual), responsáveis pela produção de aproximadamente 90% da saliva, e glândulas salivares menores, complementando os 10% restantes de fluido formado (WOYCEICHOSKI *et al.,* 2013). Nas glândulas salivares de humanos e roedores é possível observar dois componentes, os ácinos e os ductos. Sendo que os ácinos são compostos por células serosas e/ou mucosas e são responsáveis pela produção da saliva primária. Enquanto os ductos são responsáveis pela

modificação dessa saliva primária em secundária, que será secretada na cavidade oral. A secreção salivar é definida como um fluído misto – o seroso, que é produzido pelos ácinos da parótida e o misto (seroso e mucoso), produzido pelos ácinos das glândulas submandibular e sublingual (Melo, 2013). Dentre esses componentes serosos e mucosos também estão incluídas enzimas e proteínas antimicrobianas, imunoglobulinas, glicoproteínas da mucosa, fatores de crescimento e peptídeos (BHATTARAI *et al.,* 2018)

O fluxo salivar pode ser definido como a quantidade de saliva secretada em determinado período de tempo. A sua regulação, juntamente com a composição salivar, é essencialmente controlada pelo sistema nervoso autônomo simpático e parassimpático. A composição salivar secretada pelas glândulas depende dos estímulos de ambos os sistemas, de forma que, o sistema parassimpático estimula a liberação de uma secreção mais fluídica, aquosa, e uma saliva em maior quantidade de volume – isso ocorre através da ligação da acetilcolina aos receptores muscarínicos do tipo M_3 (BHATTARAI *et al.,* 20180. Já o simpático, potencializa essa estimulação parassimpática através da liberação de noradrenalina, que ao ligar-se aos receptores β-adrenérgicos, estimula a produção de uma saliva mais viscosa, por conter uma maior quantidade de proteínas e pouco volume, o que pode acarretar na sensação de boca seca (BHATTARAI *et al.,* 2018; PROCTOR e CARPENTER, 2014; C SCULLY, 2003; APS e MARTNES, 2005).

A saliva desempenha um papel de extrema importância na saúde bucal, pois auxilia na digestão e lubrificação do bolo alimentar (BHATTARAI *et al.,* 2018; AZUMAL, KATADA E SANO, 2018). A saliva também exerce um papel fundamental na modulação dos ecossistemas microbianos orais, impedindo o desenvolvimento de doenças na cavidade oral, atuando de forma protetora sobre a mucosa e dentes (ANTONIAZZI *et al.*, 2017; AZUMAL, KATADA E SANO 2018; WOYCEICHOSKI *et al.,* 2013). Além disso, possui a capacidade de tamponamento, remineralização dos elementos dentais e produção de fatores de crescimento e outros peptídeos reguladores (ANTONIAZZI *et al.,* 2017; ASSY E BRAND, 2018). E a execução dessas funções salivares depende da sua composição e do fluxo salivar, pois uma redução nesses parâmetros pode desencadear infecções orais, aumento na ocorrência de gengivites, instalação de processos cariosos, mau hálito e até implicações gastrointestinais, por conta da perda da função protetora exercida pela saliva (WOYCEICHOSKI et al., 2013; ANTONIAZZI *et al.,* 2017; SMITH E BURTNER**,** 1994; ASSY E BRAND, 2018). Pacientes com secreção salivar diminuída (hipossalivação) podem relatar um sintoma, a

xerostomia (percepção subjetiva da boca seca; ASSY E BRAND, 2018). A xerostomia pode acarretar sialose, gengivite, perda da sensação gustativa e outros distúrbios da cavidade oral, gerando um impacto negativo na qualidade de vida dos acometidos (BHATTARAI *et al.,* 2018; AZUMAL, KATADA E SANO, 2018).

Distúrbios salivares, como a redução do fluxo salivar, podem estar associados a diversos fatores, tais como: doenças sistêmicas, drogas e tratamento de radioterapia envolvendo cabeça e pescoço. Dentre esses, os que mais afetam o fluxo salivar são as drogas, tanto terapêuticas quanto as de uso recreativo e/ou aditivo (WOYCEICHOSKI *et al.,* 2013; SMITH E BURTNER**,** 1994). Como exemplos de drogas terapêuticas que afetam o fluxo salivar pode-se citar os diuréticos, laxantes, antiácidos, anorexígenos, anti-hipertensivos, antidepressivos, antipsicóticos, benzodiazepínicos, anti-histamínicos e anticolinérgicos. Enquanto as drogas ilícitas associadas a xerostomia podem-se citar os opioides, a maconha, o ecstasy, o tabaco, o álcool, a metanfetamina e a cocaína (ANTONIAZZI *et al.,* 2017; C SCULLY, 2003). Por fim, pacientes que fazem uso concomitante da cocaína ou *crack* com álcool ou tabaco apresentam maiores taxas de dentes cariados ou perdidos por patologia periodontal avançada, sendo que ambos são marcados pela xerostomia (WOYCEICHOSKI *et al.,* 2013).

1.4. Osso

Os ossos exercem funções primordiais para o corpo, promovendo a sustentação, locomoção e proteção de órgãos vitais. Apresentam-se através de dois tipos: o cortical e o trabecular (ou esponjoso). Aproximadamente 20% dos ossos são esponjosos, sendo encontrados no interior e extremidades de ossos longos. Os restantes 80% são compostos por ossos corticais, que estão presentes no esqueleto axial e em ossos longos como tíbia e fêmur. Apesar da similaridade, possuem diferentes configurações estruturais; no osso cortical são encontrados os canais de Harvers e ao seu redor são depositadas camadas cilíndricas de tecido ósseo, enquanto no osso trabecular não são encontrados esses canais e a deposição ocorre em camadas longitudinais. Dessa forma, pode-se afirmar que o osso trabecular possui uma menor rigidez e resistência, devido a essa diferença microestrutural (CHARPAD *et al.,* 2008; KANG *et al.,* 2016).

Os ossos longos, como o fêmur, apresentam três centros de ossificação: i. uma diáfise (corpo); ii. duas epífises (extremidades); e iii. duas metáfises (transição entre diáfise e a epífise). A diáfise é circunscrita por osso cortical, protegendo seu conteúdo que é a

medula óssea amarela. Já a epífise é composta em sua maioria por osso esponjoso, com porção externa fina de osso compacto (cortical) contendo a medula óssea vermelha. Na metáfise há o disco epifisário, que é uma placa de crescimento formada por cartilagem hialina, sendo substituída por uma linha epifiseal, quando o osso longo encerra seu desenvolvimento (ANDIA, CERRI E SPOLIDORIO, 2006).

O tecido ósseo é constituído por uma matriz orgânica e inorgânica, classificada conforme sua composição. A matriz orgânica é preenchida por células ósseas (osteoblastos, osteócitos e osteoclastos), fibras colágenas e substância base (proteoglicanos e glicoproteínas). Nessa matriz há uma maior concentração de colágeno tipo I, além de água e componentes minerais, como a apatita carbonatada. A matriz inorgânica é formada principalmente por fosfato de cálcio, responsável por dois terços do peso ósseo. O fosfato interage com hidróxido de cálcio formando os cristais de hidroxiapatita. Outros componentes inorgânicos também podem estar presentes, como: o carbonato de cálcio, sódio, magnésio e fluoreto (BOSKEY *et al.,* 2001; FARLAY *et al.,* 2012). Esse reservatório de íons minerais, mantém a homeostase do tecido ósseo. O conteúdo mineral está diretamente relacionado com as propriedades elásticas deste tecido e, consequentemente, com a sua resistência, determinando se o osso irá fraturar ou formar microtrincas ao ser submetido a uma força externa. Essa resistência e rigidez são garantidas pela deposição de cristais de hidroxiapatita, juntamente ao colágeno tipo I e proteínas não colágenas (FARLAY *et al.,* 2012; BOLEAN *et al.,* 2017).

As células ósseas contidas na matriz orgânica são originadas de células-tronco e se diferenciam basicamente em osteoblastos e osteoclastos. Os osteoblastos atuam secretando matriz óssea extracelular, colágeno e componentes não mineralizados e apresentam como principal função a produção de tecido ósseo. A medida que ocorre a formação dessa matriz, os osteoblastos amadurem e se incorporam a ela, diferenciando-se em uma outra célula, os osteócitos, que atuam na manutenção da matriz óssea.

Os osteócitos encontram-se individualmente nas junções lamelares (lacunas da matriz) e possuem prolongamentos que permitem a comunicação entre si através dos canalículos. Os osteócitos podem sintetizar ou absorver a matriz óssea e, a absorção ocorre nos casos em que há destrição dessa célula, e consequentemente a reabsorção óssea através da atividade osteoclástica. Esta atividade é realizada por células denominadas osteoclastos, que promovem a reabsorção óssea. Os endósteos, células osteoprogenitoras, também podem ser encontrados na camada periosteal. Os endósteos

se diferenciam em osteoblastos, permitindo a reparação óssea na região (ANDIA, CERRI E SPOLIDORIO, 2006; HARADA E HODAN, 2003).

Através dessas funções celulares os ossos podem se adaptar através dos processos fisiológicos de modelagem e de remodelação. O processo de modelagem é responsável pelo crescimento em comprimento do esqueleto, formando a massa óssea no período da infância/adolescência – nesse processo os osteoblastos exercem maior atividade que os osteoclastos. Já o processo de remodelação ocorre durante toda a vida, o osso de adapta às alterações externas, como por exemplo, hormonais e metabólicas e a partir dessas alterações uma porção óssea que tenha se tornado não funcional é reabsorvida pelos osteoclastos, dando lugar a uma estrutura óssea recém-formada por osteoblastos. Essa nova estrutura se difere em sua microarquitetura através do osso cortical, que é formado por ósteons cilíndricos que circundam os canais de Havers e por osso trabecular com curvas e ósteons incompletos (CHARPAD *et al.,* 2008; FARLAY *et al.,* 2012).

A microarquitetura óssea, seja a formada no desenvolvimento ou aquela advinda de adaptações externas ou internas, está diretamente relacionada a qualidade do osso. A mensuração da qualidade óssea é fornecida através de fatores intrínsecos, como a qualidade mineral, a qualidade do colágeno e a presença de microtrincas. Esses fatores associados a fatores extrínsecos, como a massa óssea, a densidade mineral do osso e a microarquitetura determinam a resistência óssea. Além desses fatores, a taxa de remodelação, o grau de mineralização, o tamanho dos cristais de hidroxiapatita, o colágeno e a ausência/deficiência de colágeno na composição proteica, a viabilidade dos osteócitos e a resistência nanomecânica podem interferir na qualidade óssea, provocando uma fragilidade a nível tecidual. A qualidade óssea pode ser afetada por doenças metabólicas, que antigamente eram definidas através da sua fisiopatologia apenas como uma perda de massa óssea. Atualmente, sabe-se que essas alterações ósseas comprometem a microarquitetura do tecido ósseo, acarretando em um maior riso de fraturas (CHARPAD *et al.,* 2008).

1.5. Implicações no feto devido ao uso do crack no período gestacional

Os produtos da pirólise do *crack* são metabolizados e podem atravessar a barreira placentária, já que possuem baixo peso molecular, e causar efeitos teratogênicos (MATOS *et al.,* 2011). Os metabólitos do *crack* são armazenados no miométrio e na

membrana placentária e liberados continuamente para o líquido amniótico, expondo o feto a uma liberação lenta e contínua dessas substâncias (PARCIANELLO *et al*. 2018). Além dos efeitos teratogênicos, há relatos na literatura de efeitos neurocomportamentais (MATOS *et al.,* 2011), comprometimento na estatura e no tônus muscular (WILIAMS e JOHNS, 2014), partos prematuros, anomalias cardíacas, baixo peso ao nascer, menor comprimento ósseo e menor perímetro encefálico, quando da exposição pré-natal à fumaça do *crack* (CAMPILLO *et al.,* 2004).

Diante disso, o presente trabalho teve por um dos objetivos avaliar se a exposição à fumaça do *crack* durante o período gestacional interfere no desenvolvimento e qualidade óssea das proles. Além disso, avaliar o fluxo e a composição salivar nesses animais (ratos Wistar machos). Até o presente momento, no conhecimento dos autores, não existem descrições na literatura sobre as alterações morfofuncionais óssea e salivar promovidas pela exposição à fumaça do *crack* durante o período intrauterino.

2. CAPÍTULOS

ARTIGO 1 - Wistar rats exposed to crack cocaine during the intrauterine period do not present changes in salivary flow rate and composition until one month after birth

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Abstract

The use of additive substances during the gestational period is an important social and public health problem. In recent years attention has been drawn to the indiscriminate use of crack cocaine in pregnancy. Crack cocaine has deleterious effects on the mother and newborn, including cardiac abnormalities, low birth weight, shorter bone length and lower brain perimeter. The objective of the present study was to evaluate the effects on the salivar flow rate and composition of animals exposed to crack cocaine during the intrauterine period. For this, pregnant Wistar rats were exposed during the 5th and 21st days of the gestational period to crack cocaine for 5 min. Control animals remained in the housing boxes during the exposure period. Postnatal day 30, male rats exposed to crack cocaine during the intrauterine period and control were anesthetized. After confirmation of the anesthesia, the animals were treated with the sialogenic agent pilocarpine (non-selective muscarinic agonist, 2 mg/kg, i.p.) and then the salivary secretion stimulated was collected for 10 min. At the end, the animals were sacrificed and the salivary glands (parotid, submandibular and sublingual) were collected and weighed. The composition of the saliva was analyzed by Fourier transform infrared spectroscopy (FTIR). Our results did not show significant differences in the flow rate and weight of the salivary glands, as well as in the amide, nitrogenous bases, amide I, proteins and sugar fractions between the experimental groups ($P > 0.05$). Thus, our results indicate that exposure to crack cocaine during the intrauterine period does not alter the salivary flow rate and composition of offspring one month after birth.

Key-words: Gestational Crack cocaine, Bone, Saliva, FTIR.

1. Introduction

Cocaine is derived from leaves of *Erythroxylum coca*, and is a central nervous system stimulant drug. Cocaine can be administered orally, intravenously or inhaled (PARCIANELLO *et al.,* 2018; FREITAS *et al.,* 2014). Crack cocaine has more deleterious effects than cocaine used intranasally or injected. While cocaine takes about fifteen minutes to reach the central nervous system, crack takes only eight to fifteen seconds and its effect lasts for about ten minutes, consequently the effects of crack are more rapid and intense, as well as the signs of dependence (FISCHMAN, 1988).

Crack cocaine dependence is a worldwide public health problem. Crack pyrolysis products are able to cross the placental barrier, causing teratological effects (MATOS *et al.,* 2011), that including, learning and memory deficits (ANDRET *et al.,* 19996), increased probability of acquiring cocaine self-administration during adulthood (ROCHA et al., 2002), impairment of height and muscle tone (WILIAMS and JOHNS, 2014), premature births, cardiac anomalies, low birth weight, and lower brain perimeter (CAMPILLO, 2004). An important effect of crack cocaine use that is also observed is hyposalivation (ROCHA *et al.,* 2002; WOYCEICHOSKI *et al.,* 2013).

Saliva is a biological fluid formed by secretions produced by the major salivary glands (parotid, submandibular and sublingual), responsible for the production of approximately 90% of saliva, and smaller salivary glands (WOYCEICHOSKI *et al.,* 2013). Salivary flow rate is the amount of saliva secreted in a given period of time and its regulation, along with the salivary composition, is essentially controlled by the autonomic sympathetic and parasympathetic nervous system (BHATTARAI et al., 2018; PROCTOR and CARPENTER, 2014; SCULLY, 2003; MARTENS, 2005).

Saliva exerts several functions, in digestion it helps the lubrication of the food bolus, facilitates mastication and swallowing, and food and bacterial clearance (BHATTARAI *et al.,* 2018; AZUMA *et al.,* 2018). Saliva also plays an importante role in the modulation of oral microbial ecosystem, preventing the development of diseases in the oral cavity (WOYCEICHOSKI *et al.,* 2013; AZUMA *et al.,* 2018; ANTONIAZZI *et al.,* 2017). In addition, saliva performing functions such as buffering capacity, remineralization of dental elements and production of growth factors and regulators (ANTONIAZZI *et al.,* 2017; ASSY and BRAND, 2018).

However, in order for saliva to perform its various functions, control of salivary flow rate and composition is necessary. In fact, an alteration in both parameters can cause an

imbalance in their protective function, triggering oral infections, increased gingivitis, carious processes, bad breath and even gastrointestinal implications as xerostomia (ANTONIAZZI *et al.,* 2017; ASSY and BRAND, 2018; SMITH and BURTNER, 1994).

Saliva disorders such as reduced salivary flow rate may be associated with several factors, such as systemic diseases, therapeutic and recreational drugs, treatment of head and neck radiation therapy (WOYCEICHOSKI *et al.,* 2013; SMITH and BURTNER, 1994). Among the illicit drugs associated with xerostomia are opioids, cannabis, ecstasy, tobacco, alcohol, methamphetamine and cocaine (SCULLY, 2003; ANTONIAZZI *et al.,* 2017). Patients who use concomitant cocaine or crack cocaine with alcohol or tobacco have higher rates of decayed or lost teeth, advanced periodontal disease, both marked by xerostomia (WOYCEICHOSKI *et al.,* 2013).

Therefore, this study aims to evaluate the salivary flow rate and composition of young rats exposed to crack cocaine during the intrauterine period. Our hypothesis is that exposure to crack smoke in the intrauterine period alters salivary flow and composition in young rats.

2. Methods

2.1. Experimental design

The present study was approved by the Committee on Ethics in the Use of Animals (CEUA/UFAL) project N° 50/2016. Male and female Wistar rats, aged between 60-90 days, were obtained from the Central Biotério da UFAL (BIOCEN/UFAL).

2.1.1. Mating of animals

Thirty-two Wistar rats (32 = 16 males and 16 females) were used for mating. The animals were mated in a ratio of 1:1, using the Poiley method, which consists of the rigid cross-over scheme with predetermined groups (POILEY, 1960), aiming at maintaining the heterozygosity of the descending population.

Initially, the males were distributed in individual cages, after 24 hours the males were removed, and the females were inserted in the same cages without being cleaned, aiming to obtain the *Whitten* effect, caused by the action of the pheromones produced by the male

rodents (SANTOS, 2002). At 5:00 p.m. the males were housed in conviviality with the respective females and removed at 7:00 a.m. the next day. To confirm copulation, at 7:00 a.m. was lavage performed in the vaginal canal of the female with 20 μL of 9% sodium chloride solution using a pipette. The material harvested was placed in glass slides and smeared for microscopic verification, if sperm were present (MARCONDES *et al.,* 2002), that day was considered to be day 0. If not, the male was reinserted at 5:00 p.m. again with the female, for a new attempt and later analysis the following morning at 7:00 a.m.; being repeated this phase of copulation until four consecutive days.

2.1.2. Exposure to crack smoke

After confirmation of pregnancy, rats (n = 12) were divided into two groups: Group 1 - exposed to crack cocaine for 5 minutes during the gestational period (n = 6 rats); and Group 2 - the control group, do not exposed to crack cocaine during the gestational period (n = 6 rats). G1 rats (n = 6) were exposed to crack cocaine from the 5th day of gestation at 21th, daily in the morning. The females remained with their offspring until weaning and sexing, which occurs around the 21st day of life.

For the exposure of the pregnant rats to the drug, the apparatus consists of a set fan for generation of 150 ml/min (VETERINARY ANESTHESIA VENTILATOR MODEL, 2000, Hallowell EMC, Pittsfield, MA). A smoke-generating pump is attached at one end to a pipe (where the substance will be burned) and in the other to an acrylic chamber (where the animals are housed). The boxes are serially connected by silicone tubes interconnected by heimlich valves (Becton Dickinson, Franklin Lakes, NJ), to prevent regression of smoke. The chamber has an upper cover and two sets of smoke elimination holes on the side opposite the tube where it will be supplied and these holes can be blocked with two acrylic rectangles contained in the system.

The protocol used to burn the drug was performed according to the protocol pre-established (SILVA, 2016), developed by our own laboratory. The protocol consists of placing the animal in the fully closed acrylic box for one minute for ambiance; then the smoking pipe is heated through the use of a torch for two minutes; after heating the crack rock (200mg) is inserted into the smoking pipe, the apparatus is turned on and the drug is burned for two minutes, through the smoke suction system that is produced by the burning is thrown in the acrylic box where the animal; being exposed to crack smoke for 5 minutes with the box fully closed and another 5 minutes providing oxygen by removing the acrylic

rectangles exposing the contralateral holes, allowing the smoke to escape to the exhaust fan and the oxygen inlet. During the handling of the substance, the investigators were properly equipped with the respective personal protective equipment (PPE): specific clothing for handling chemical agents, caps and respirator with appropriate filters.

The crack cocaine samples were obtained through a judicial order, with the release of ANVISA, at the narcotics police station, Maceió-AL. They were submitted to the chromatographic analysis and it was possible to identify the crack smoke composition through gas chromatography coupled to mass spectrometry, in which it was possible to identify methylecgonine, also known as methyl ester anhydroecgonine (AEME) and benzoic acid, are compounds derived from pyrolysis or thermal decomposition of cocaine (ARAÚJO *et al.,* 2018). The compound AEME is generated exclusively from the pyrolysis of cocaine and is therefore an analytical marker of crack use (WOOD *et al.,* 2007; TOENNES *et al.,* 2003). By means of this previous analysis of the samples it was possible to affirm that they contained cocaine / crack metabolites, thus being viable to be used in this study.

2.1.3. Birth and Sexing of offspring

After birth, the number of pups from each rat was counted and weighed daily. On the 21st day of the offspring's life, sexing was performed. Where the males were packed in acrylic boxes with chromed iron cover, subdivided into groups of no more than 5 animals/box. Waiting until the 30th day of life to collect the biological samples.

2.2. Extraction of biological materials

Two male pups were used per group 1 and group 2 rats (Group 1: 6 exposed rats → 12 pups) (Group 2: 6 control rats → 12 pups), and the pups from each group were subdivided according to the analysis.

2.2.1. Extraction of salivary secretion and salivary glands

On the 30th day of life, animals (G1A; n = 6 and G2A n = 6) were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (12 mg/kg, i.p.). After confirming the anesthesia, the animals were treated with pilocarpine (2 mg/kg; i.p.) - model of

stimulation of salivary secretion stimulated (TAKAI *et al.,* 1983). The salivary secretion was collected for 10 minutes with the aid of a 10 µl pipette and stored in an eppendorf being weighed shortly after collection; then the parotid, submandibular and sublingual salivary glands were collected, which were also weighed and stored in eppendorfs. Saliva and salivary glands were stored at -20 ° C for further analysis of salivary secretion and saliva chemical components through Fourier transform infrared spectroscopy (FTIR).

2.3. Analysis of biological materials

2.3.1. Salivary flow

Analysis of salivary flow per gram of glandular tissue is the ratio of the volume of salivary secretion to the sum of the weight of the salivary glands.

2.3.2. Weight gland saliva

The parotid, submandibular and sublingual salivary glands were dissected and weighed on an analytical scale.

2.3.3. FTIR

. Samples were thawed one hour prior to the start of the experiment (stored at 20c) and stripped for analysis. The chemical profile recorded from 3000-400 cm^{-1}, through the Vertex 70 FTIR spectrophotometer (Bruker, Billerica, Massachusetts, USA) associated with a total reflectance microtitre (ATR) accessory, a diamond disc as a reflection element internal; the temperature during recording was (23 ± 1 ° C), room temperature. The normalization and baseline of the spectra of the samples were corrected through OPUS 6.5 software (Bruker, Billerica, Massachusetts, USA) (KHAUSTOVA *et al.,* 2010).

2.4. Statistical analysis

All values reported as mean ± SEM. The comparisons of the means were performed by the unpaired Student t-test and non-parametric Mann-Whitney test

(GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA). Values of p <0.05 were considered statistically significant.

3. Results

3.1. Salivary flow

The salivary flow of the rats exposed to crack cocaine in the gestational period and the control rats are represented in figure 1.

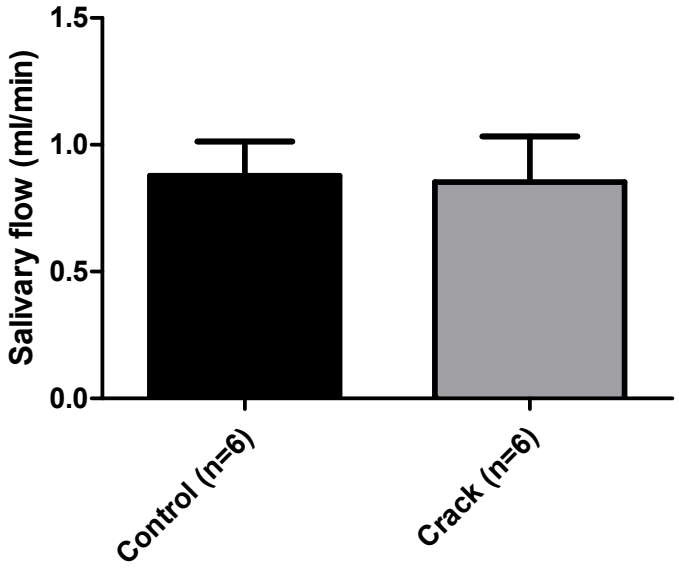

Figura 1. Salivary flow results. Salivary flow rate evaluated in the control (n = 6) and crack cocaine rats (n = 6)**.** Analyzed by t-Student parametric test, results are Mean ± SEM (p > 0.05).

3.2. Weight salivary glands

The weight of the salivary glands of rats exposed to crack cocaine in the gestational period and control rats were obtained, but there was no difference between the groups (*P* > 0.05) (Figure 2A-C).

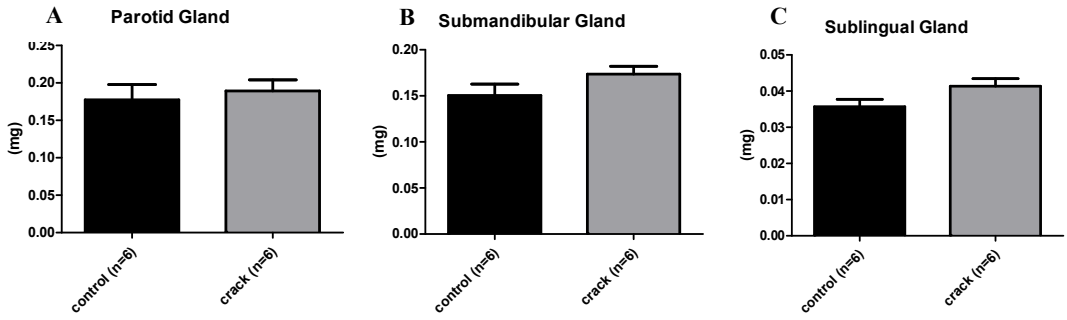

Figure 2. Weight salivary glands. Control (n=6) and Crack rats (n = 8). A. Mean ± SEM of weight parotid gland, analyzed by the non-parametric Mann-Whitney test ($p > 0.05$). B. Mean ± SEM of weight submandibular gland analyzed by t- Student parametric test ($p > 0.05$). C. Mean ± SEM of weight sublingual gland, analyzed by the non-parametric Mann-Whitney test ($p > 0.05$).

3.3. FTIR

The spectrum obtained from the saliva of rats exposed to crack cocaine in the gestational period and from the control rats are shown in Figure 3. The bands at 3358 cm^{-1}, 1638 cm^{-1}, 1557 cm^{-1}, 1385 cm^{-1} and 1033 cm^{-1} are identified as amide A, amide I, proteins and sugars moieties, respectively (table 1), these components remained unchanged ($P > 0.05$) at crack compared to control rats (Figure 4A-E). The band area of the 671cm^{-1} was identified as the only one with altered parameters ($P < 0.05$) compared to control rats (Figure 4F).

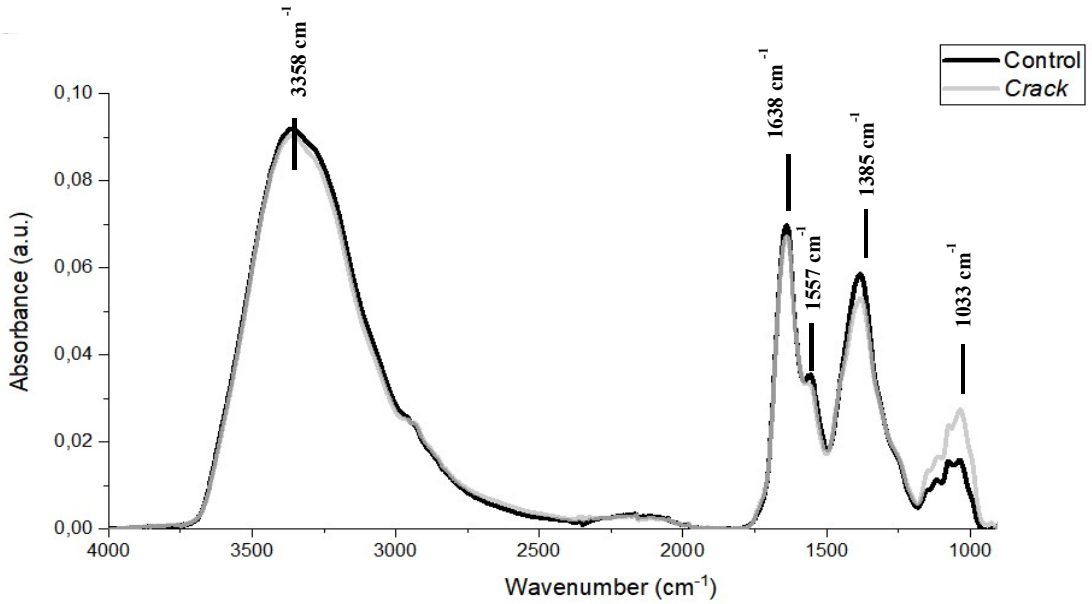

Figure 3. FTIR spectrum of saliva. Mean spectrum of salivar components obtained by the FTIR analysis in Control and Crack cocaine rats.

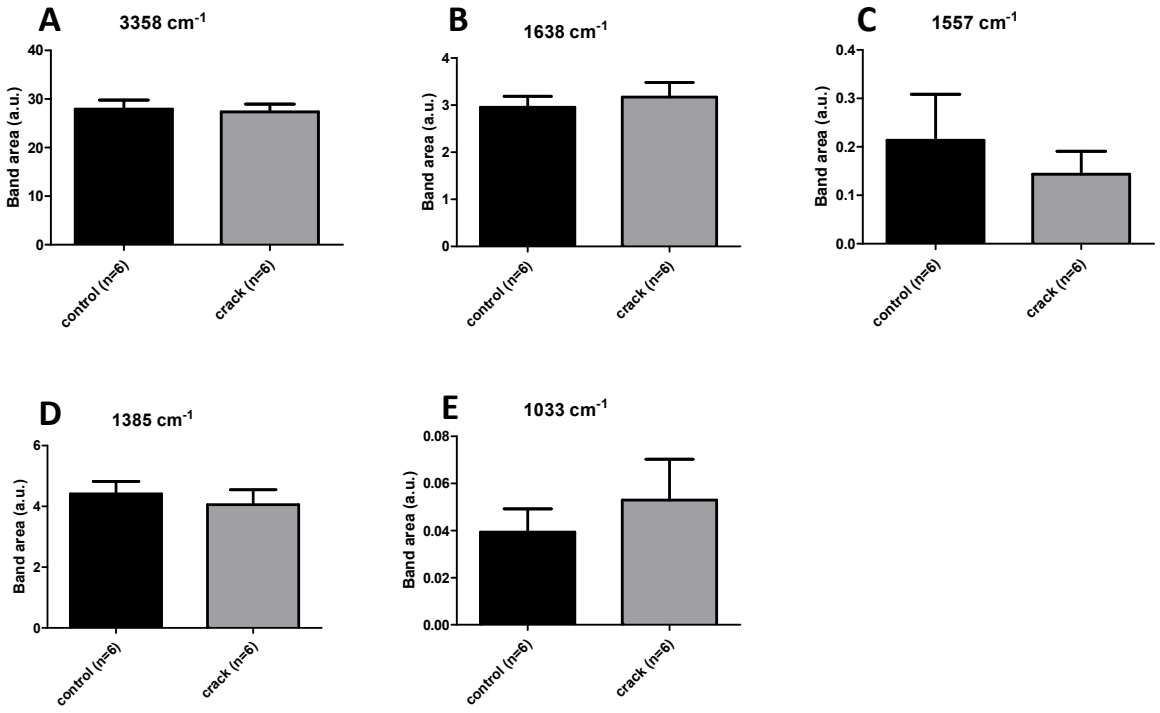

Figure 4. FTIR compositional results. Salivar components evaluated in Control (n=6) and Crack rats (n = 8). A. Mean ± SEM of percentage of Amide A (368 cm-1) analyzed by t-Student parametric test ($p > 0.05$). B. Mean ± SEM of Amide I (1557 cm-1) analyzed by t-Student parametric test ($p > 0.05$). C. Mean ± SEM of the proteins (1385 cm-1) analyzed by t- Student parametric test ($p > 0.05$). D. Mean ± SEM of Sugar moieties (1033 cm-1) analyzed by t- Student parametric test ($p > 0.05$).

Table 1. Vibrational modes and identification of the respective salivary component.

Band Frequency (cm^{-1})	Peak Frequency (cm^{-1})	Component Identification	Vibrational Mode
3680-2990	**3358**	**Amide A (27)**	**Symmetric N-H stretching**
	1638	**Nitrogen Bases**	**C=C thymine, adenine, N-H guanine (28)**
1730-1500	**1557**	**Amide I (α-helice) (27)**	**C=O stretching**
1427-1368	**1385**	**Proteins (29)**	**Symmetric CH3 bending**
1190-1000	**1033**	**Sugar moieties (27)**	**CH2OH groups, C-O stretching & COH groups bending, symmetric PO2- stretching**

4. Discussion

In the present study, no change in salivary secretion stimulated by pilocarpine was observed in young rats exposed to crack smoke during the gestational period compared to the control group. Data observed in the literature describe the relationship between salivary flow and crack, in users only, no studies were found that performed this association in the children of crack users. A research was observed that after a salivary collection of 54 users of crack in comparison to saliva of non-users, in which among the parameters analyzed was the salivary flow, that the same remained unchanged between groups. However, recent studies with more defined exclusion criteria observed that crack users had a reduced salivary flow compared to the non-users group (WILIAMS and JOHNS, 2014); in another larger study it was observed that users of illicit drugs, among them crack, had lower salivary flow than the control group (SORDI, 2017).

Hyposalivation due to drug use may be related to local and systemic factors. The local factor may be due to vasoconstriction that is related to a reduction in salivary flow.

The systemic factor can be exemplified by the users of crack is explained pharmacologically, since this drug is a stimulant of the central nervous system, interacting with catecholamines, norepinephrine and dopamine, blocking the presynaptic reception, consequently making available concentrations of these neurotransmitters in post receptors synaptic. Thus, high concentrations of noraepinephrine will act on the sympathetic nervous system, which is responsible for the production of saliva in a small amount, allowing the sensation of dryness in the mouth (PARCIANELLO *et al.,* 2018). Although this pharmacological explanation covers crack users, it would not be possible to relate this same pharmacology to the children of users, justifying that no changes in salivary flow patterns were observed.

A review about the relationship between salivary flow reduction and obesity has raised the hypothesis that parotid gland size might influence hypofunction and consequent hyposalivation, but this association has not been well understood (FREITAS *et al.,* 2014). In our research, we did not identify significant differences between the salivary gland sizes of the exposed group in relation to the control group.

Alterations in the spectral profile of the saliva of crack rats could suggest absence or deficiency of some component, causing problems of functional saliva performance, such as its immunoprotective effect or its buffer capacity (WILIAMS and JOHNS, 2014; CAMPILLO, 2004). Consequently causing problems in oral health. In addition, some component could be found that could be used as a biomarker, indicating the presence of crack components from exposure in the gestational period. As there are reports in the literature of salivar biomarkers used for cancer diagnosis (MOVASAGHI, 2008); dementia (LOPES, 2016); Stress (JR CAETANO, 2015).

The groups are already in the literature through spectral analysis, the problem of identification and even the diagnosis of chronic diseases, through the differences between the processes in the graph analysis. Among the wavelengths of wired waves, observing that the peaks 3358cm^{-1}; 1638 cm^{-1}, are representative of the AMIDA group and can be correlated with Urea. Studies have shown that urea levels in experimental salivary model for the brain nephropathies patients (BILANCIO, 2018) and patients with acute renal disease (KOVALCIKOVA, 2018) However, it was not able to make the difference between the crack and control groups.

Peak 1385 cm^{-1}, is related to proteins, as they are cited in Immunoglobulin A (IgA) found in saliva. This antibody acts to protect the oral cavity against viruses, bacteria and other invasive microorganisms (COLOMBO, 2016). A relationship between

IgA and Streptococcus mutans (SM) (major pathogens of the carious process) has been demonstrated in children, so that children who presented high levels of MS and low IgA concentrations (COLOMBO, 2017). In another study, an inverse proportion of IgA and a vunerability to oral cavity pathologies were also observed; Influenza HIV Infectious infections by HIV were more serious than the oral, subcutaneous mucosae (SUBRAMANIAM and KUMAR, 2103). In the results of the results, the difference between the groups, in order to protect the protrusion, was not performed, since the IgA was not applied to that of the age of 30 days.

The wavelength represented by the $1033cm^{-1}$ peak represents portions of sugar which may be corroded with a glucose. It has been shown that salivary glucose is a tool that serves as a biomarker for the diagnosis of diabetes (TIONGCO *et al.,* 2018). This peak was also not significantly evaluated between the control group and the group exposed in the gestational period.

It may be suggested that crack metabolites that cross the placental barrier do not influence the development of salivary glands, morphological aspects, nor the production of salivary secretion, neither in its flow nor in its composition. Thus, oral changes in children / youngsters who were exposed to crack during the intrauterine phase would not be related to a hyposalivation resulting from the crack effect during pregnancy.

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2. CAPÍTULOS

ARTIGO 2 - Changes in bone microarchitecture and mineral composition in young rats exposed to crack cocaine in intrauterine period

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Abstract

The use of additive substances during the gestational period is an important social and public health problem. In recent years attention has been drawn to the indiscriminate use of crack cocaine by pregnant women. Crack cocaine has deleterious effects on the mother and newborn, including cardiac abnormalities, low birth weight, shorter bone length and lower brain perimeter. The objective of the present study was to evaluate the effects on the composition and microarchitecture of the bone tissue (femur) of animals exposed to crack cocaine during the intrauterine period. For this, pregnant Wistar rats were exposed during the 5th and 21st days of the gestational period to crack cocaine for 5 min. Control animals remained in the housing boxes during the exposure period. Postnatal day 30, male rats exposed to crack cocaine during the intrauterine period and control were sacrificed for the removal of the right femur and subsequent analysis of the chemical profile and bone microarchitecture using Fourier transform infrared spectroscopy (FTIR) and microtomography (Micro-CT), respectively. Our results demonstrated that exposure to crack cocaine did not significantly alter the content of fatty acid esters, amide I and II groups, protein curves by methylene, phosphate I, collagen and carbonate when compared to the control group. However, the CH3 symmetrical conformation was increased in the crack cocaine group. In the Micro-CT assay, there was no significant difference between the experimental groups for the trabecular bone parameters ($p > 0.05$). On the other hand, cortical bone parameters were increased in the crack group, which obtained a larger fractal dimension ($p < 0.05$) and higher number of closed pores ($p < 0.01$), purchased from the control group. Thus, our results indicate that exposure to crack cocaine during the intrauterine period increased bone quality parameters, possibly as a protective factor against crack metabolites.

Key-words: Gestational Crack, Crack babies; Bone, Saliva, FTIR, MictoCT.

1. Introduction

Cocaine and crack cocaine are a worldwide public health problem. The use of these substances by pregnant can have many serious side effects in newborns. Recently, our group published a systematic review and meta-analysis showing the consequences associated with the use of crack cocaine during the gestational period, that including, low birth weight, preterm delivery and reduced head circumference (Dos SANTOS *et al.,* 2018). In preclinical studies, rodents with fetal cocaine exposure have learning and memory deficits (ANDRET, MINNES and SINGER, 1996), increased probability of acquiring cocaine self-administration during adulthood (BEATRIZ *et al.,* 2002) and shorter bone length (CAMPILO *et al.,* 2004)

Bones perform primordial functions for the body, promoting the sustenance, locomotion and protection of vital organs (CHAPPARD *et al.,* 2008; KANG *et al.,* 2016; ANDIA, CERRI and SPOLIDORIO, 2006). The bone tissue consists of an organic and inorganic matrix. The organic component is composed of bone cells, collagen fibers (mainly type I collagen), mineral components (carbonated apatite) and water. While the inorganic matrix is formed mainly by calcium phosphate that interacts with calcium hydroxide forming the crystals of hydroxyapatite (BOSKEY et al., 2002; FARLAY and BOIVIN, 2012).

The bones can adapt through the physiological processes of modeling and remodeling. The modeling process is responsible for bone growth in the childhood/adolescence period. While the remodeling process takes place throughout a lifetime (CHAPPARD *et al.,* 2008; FARLAY and BOIVIN, 2012). Bone microarchitecture is directly related to bone quality. Several intrinsic factors, such as the presence of microtrins, and extrinsic, such as bone formation, can be used to measure bone quality. In addition, bone quality may be hampered by metabolic diseases, which may increase the risk of fractures (CHAPPARD *et al.,* 2008; FARLAY and BOIVIN, 2012).

Two techniques can be used to analyze bone quality, micro computed tomography (Micro-CT) and Fourier-transform infrared spectroscopy (FTIR). Micro-CT has been reported as gold standard for analysis in animal models, including murine model (BOLEAN *et al.,* 2017). While the FTIR for analysis of the chemical components present in the bone matrix (BOLEAN *et al.,* 2017).

As far as we know, there is no report in the literature on the bone quality of young animals, including humans, exposed to crack cocaine in the gestational period. Therefore, this study aims to evaluate the bone quality, through the microarchitecture and mineral composition, of young rats exposed to crack cocaine during the gestational period.

2. Methods

2.1. Experimental design

The present study was approved by the Committee on Ethics in the Use of Animals (CEUA / UFAL), project N ° 50/2016. Male and female Wistar rats, aged between 60-90 days, were used from the Central Biotério da UFAL (BIOCEN / UFAL).

2.1.1. Mating of animals

Thirty-two Wistar rats (32 = 16 males and 16 females) were used for mating. The animals were mated in a ratio of 1: 1, using the Poiley method, which consists of the rigid cross-over scheme with predetermined groups (POILEY , 1960), aiming at maintaining the heterozygosity of the descending population.

Initially, the males were distributed in individual cages, after 24 hours the males were removed, and the females were inserted in the same cages without being cleaned, aiming to obtain the *Whitten* effect, caused by the action of the pheromones produced by the male rodents (SANTOS, 2002). At 5:00 p.m. the males were housed in conviviality with the respective females, and removed at 7:00 a.m. the next day. To confirm copulation, at 7:00 a.m. was lavage performed in the vaginal canal of the female with 20 μL of 9% sodium chloride solution using a pipette. The material harvested was placed in glass slides and smeared for microscopic verification, if sperm were present (MARCONDES, BIANCHI and TANNO, 2002) if that day was considered to be day 0. If not, the male was reinserted at 5:00 p.m. again with the female, for a new attempt and later analysis the following morning at 7:00 a.m.; being repeated this phase of copulation until four consecutive days.

2.1.2. Exposure to crack smoke

After confirmation of pregnancy, rats (n = 12) were divided into two groups: Crack - exposed to crack smoke during the gestational period (n = 6 rats); and Control - not exposed to crack smoke during the gestational period (n = 6 rats). Crack rats were exposed to crack smoke from the 7th day of gestation at 21th, daily in the morning. The females remained with their offspring until weaning and sexing, which occurs around the 21st day of life.

For the exposure of the pregnant rats to the drug, the apparatus consists of a set fan for generation of 150 ml / min (VETERINARY ANESTHESIA VENTILATOR MODEL, 2000, Hallowell EMC, Pittsfield, MA). A smoke-generating pump is attached at one end to a pipe (where the substance will be burned) and in the other to an acrylic chamber (where the animals are housed). The boxes are serially connected by silicone tubes interconnected by heimlich valves (Becton Dickinson, Franklin Lakes, NJ), to prevent regression of smoke. The chamber has an upper cover and two sets of smoke elimination holes on the side opposite the tube where it will be supplied and these holes can be blocked with two acrylic rectangles contained in the system.

The protocol used to burn the drug was performed according to the protocol pre-established (SILVA, 2016) developed by our own laboratory. The protocol consists of placing the animal in the fully closed acrylic box for one minute for ambiance; then the smoking pipe is heated through the use of a torch for two minutes; after heating the crack rock (200mg) is inserted into the smoking pipe, the apparatus is turned on and the drug is burned for two minutes, through the smoke suction system that is produced by the burning is thrown in the acrylic box where the animal; being exposed to crack smoke for 5 minutes with the box fully closed and another 5 minutes providing oxygen by removing the acrylic rectangles exposing the contralateral holes, allowing the smoke to escape to the exhaust fan and the oxygen inlet. During the handling of the substance, the investigators were properly equipped with the respective personal protective equipment (PPE): specific clothing for handling chemical agents, caps and respirator with appropriate filters.

The crack samples were obtained through a judicial order, with the release of ANVISA, at the narcotics police station, Maceió-AL. They were submitted to the chromatographic analysis and it was possible to identify the crack smoke composition through gas chromatography coupled to mass spectrometry, in which it was possible to identify methylecgonine, also known as methyl ester anhydroecgonine (AEME) and benzoic acid, are compounds derived from pyrolysis or thermal decomposition of cocaine (ARAÚJO, *et al.,* 2018). The compound AEME is generated exclusively from the

pyrolysis of cocaine and is therefore an analytical marker of crack use (WOOD *et al.,* 2007; TOENNES *et al.,* 2003) By means of this previous analysis of the samples it was possible to affirm that they contained cocaine / crack metabolites, thus being viable to be used in this study.

2.1.3. Birth and Sexing of offspring

After birth, the number of pups from each rat was counted and weighed daily. On the 21st day of the offspring's life, sexing was performed. Where the males were packed in acrylic boxes with chromed iron cover, subdivided into groups of no more than 5 animals / box. Waiting until the 30th day of life to collect the biological samples.

2.2. Extraction of biological materials

Two male pups were used per group 1 and group 2 rats (Group 1: 6 exposed rats → 12 pups) (Group 2: 6 control rats → 12 pups), and the pups from each group were subdivided according to the analysis.

2.2.1. Femur Extraction

At the 30th day of life, the animals were anesthetized with ketamine (80 mg / kg, i.p.) and xylazine (12 mg / kg, i.p.). After that, the right femurs were removed for several analysis. The right was used for chemical components analysis through FTIR and structural measurements by Microtomography (MicroCT).

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2.3. Analysis of biological materials

2.3.1. FTIR

The right femur diaphysis were used to evaluate the bone chemical profile.Samples were stored at -20 C, the distal diaphysis were macerated and lyophilized to obtain sample spectra. The registered chemical profiles of 4000-400 cm^{-1} was obtained in Vertex 70 FTIR spectrophotometer (Bruker, Billerica, Massachusetts, USA) associated with a micro-attenuated total reflectance (ATR) accessory. Samples spectrum were recorded at room temperature (23±1 °C) with 32 scans and 4 cm^{-1} of resolution.The normalization and baseline correction was performed in OPUS 6.5 software (Bruker, Billerica, Massachusetts, USA).

2.3.2. Microtomography - Micro-CT

To evaluate the bone microarchitecture of the femur, the proximal segments of the right femurs were used. Three parts of each sample, the femoral head, the femoral neck (both for trabecular bone analysis) and the femoral diaphysis (for analysis of the cortical bone) were analyzed using a commercially available Micro - CT table system available as SkyScan 1272 (Bruker, Kontich, Belgium). During the scan the samples were wrapped by gauze moistened with water and wrapped in an eppendorf tube. The scanning parameters were 6 μm pixel size, 50 kV x-ray voltage, 160 mA electric current and 0.5 mm aluminum filter. The scanned images were then quantified using the automated image analysis system CTAn3 (Bruker, Kontich, Belgium). For this, the region of interest (ROI) was determined, comprising 400 slices of the cortical bone (femoral body) and 400 slices of the trabecular bone, 200 slices encompassing the femoral head and 200 slices of the femoral neck. In the cortical architecture were evaluated: Percentage of bone volume (BV / TV)%; Fractal dimension (FD), Closed porosity [PON (cl)]%; Degree of anisotropy (DA). And in the trabecular bone besides the TV; BV; BV / TV; BS / BV and DA; were also analyzed: Trabecular thickness (TbTh) mm; Trabecular number (TbN) mm; Trabecular separation (TbSP).

2.4. Statistical analysis

All values reported as mean ± SEM. The comparisons of the means were performed by the unpaired Student t-test and non-parametric Mann-Whitney test (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. FTIR

The femur infrared spectrum of control and crack rats are represented in figure 1. We point out 8 vibrational modes in femur of control and crack rats.

The band area at 1745 cm^{-1} was identified as bond of esters group (C= O) from fatty acids, 1644 cm^{-1} was identified as amide I, 1537 cm^{-1} as amide II, and 1451 cm^{-1} was identified as Methylene bending of proteins, Crack did not change this parameter ($p > 0.05$) compared to the control rats (Figure 2A-D). The band area at 1402 cm^{-1} indicates the CH3 symmetric deformation. This bone component was increased ($p < 0.05$) in crack than in controls rats (Figure 2E). Phosphate I is identified in the band area at 1237 cm^{-1}. This component was not affected by crack exposition ($p > 0.05$) (Figure 2F). The band area at 1030 cm^{-1} was identified as Collagen, Crack did not change this parameter ($p > 0.05$) compared to the control rats (Figure 2G). The band area at 871 cm^{-1} determines carbonate, this mineral component was not affected in crack exposed rats in relation to control rats ($p > 0.05$) (Figure 2H).

Carbonate substitution, degree of mineralization (phosphate/amide I), mineral crystallinity and collagen maturity were not altered in the femur of crack rats compared to control rats (Figure 3A-D) ($p > 0.05$).

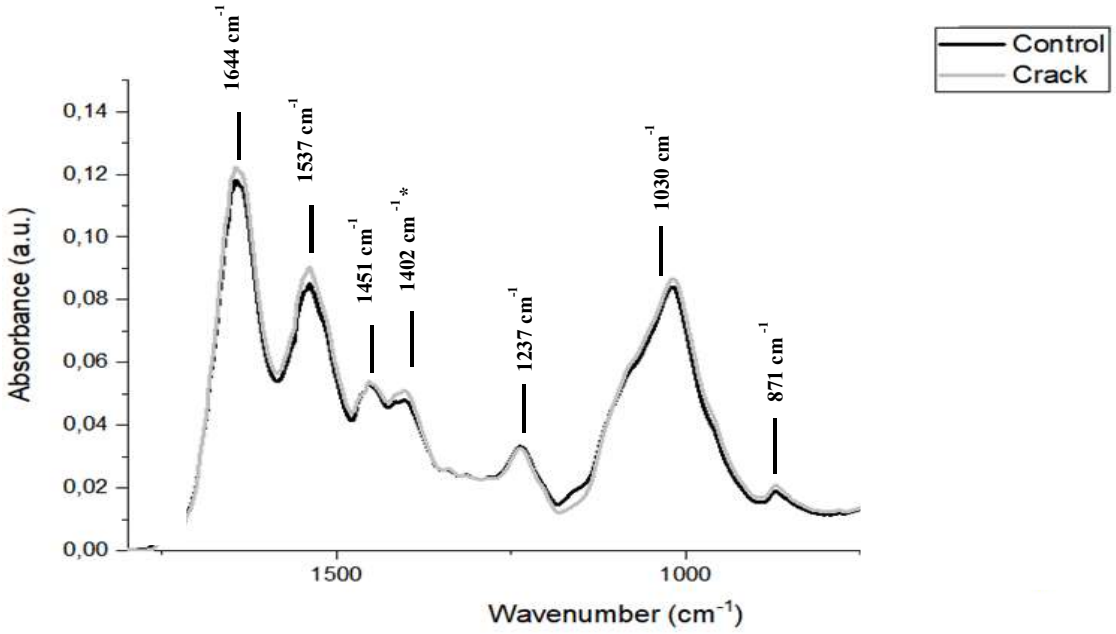

Figura 1. Espectrum of FTIR of bone. Mean spectrum of bone components analyzed by FTIR analysis in crack and control rats

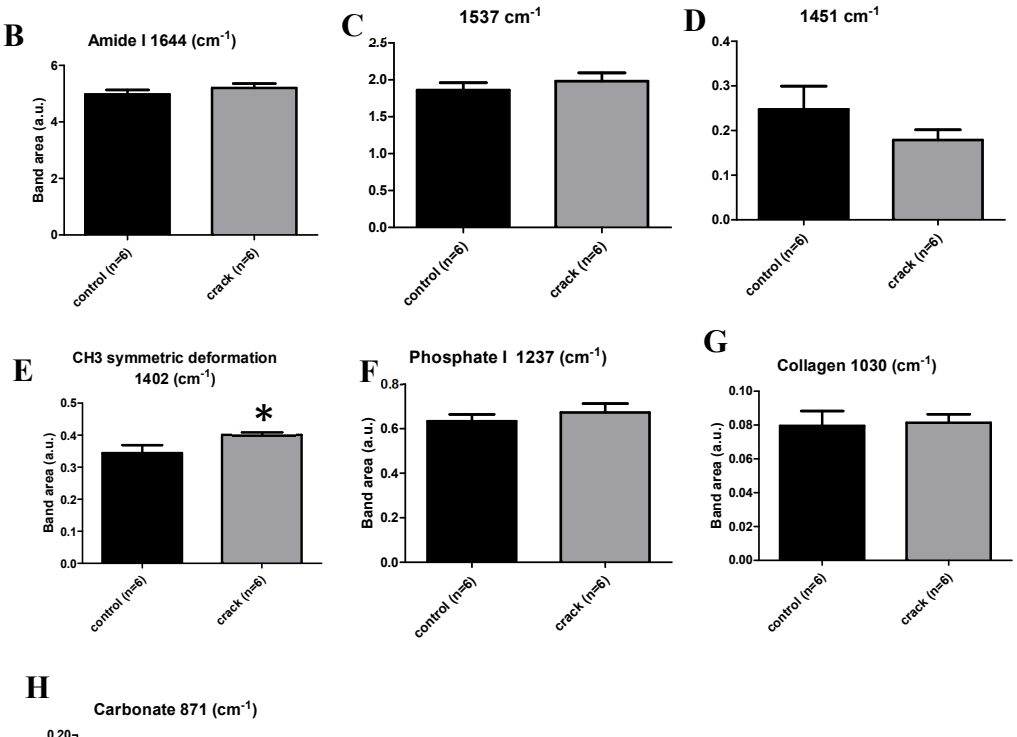

Figure 2. FTIR compositional results. Femur components evaluated in Control (n=6) and Crack rats (n = 6). **A.** Mean ± SEM of percentage of Amide I (1644 cm^{-1}) analyzed by t-Student parametric test ($p < 0.05$). **B.** Mean ± SEM of (1537 cm^{-1}) analyzed by t-Student parametric test ($p < 0.05$). **C.** Mean ± SEM of (1451 cm^{-1}) analyzed by t-Student parametric test ($p < 0.05$). **D.** Mean ± SEM of CH3 symetric (1402 cm^{-1}) analyzed by t- Student parametric test (*$p < 0.05$). **E.** Mean ± SEM of Phospahte I (1237 cm^{-1}) analyzed by t-Student parametric test ($p > 0.05$). **F.** Mean ± SEM of Collagen (1030 cm^{-1}) analyzed by t-Student parametric test ($p > 0.05$). **G.** Mean ± SEM of Crabonate (871 cm^{-1}) analyzed by t-Student parametric test ($p > 0.05$).

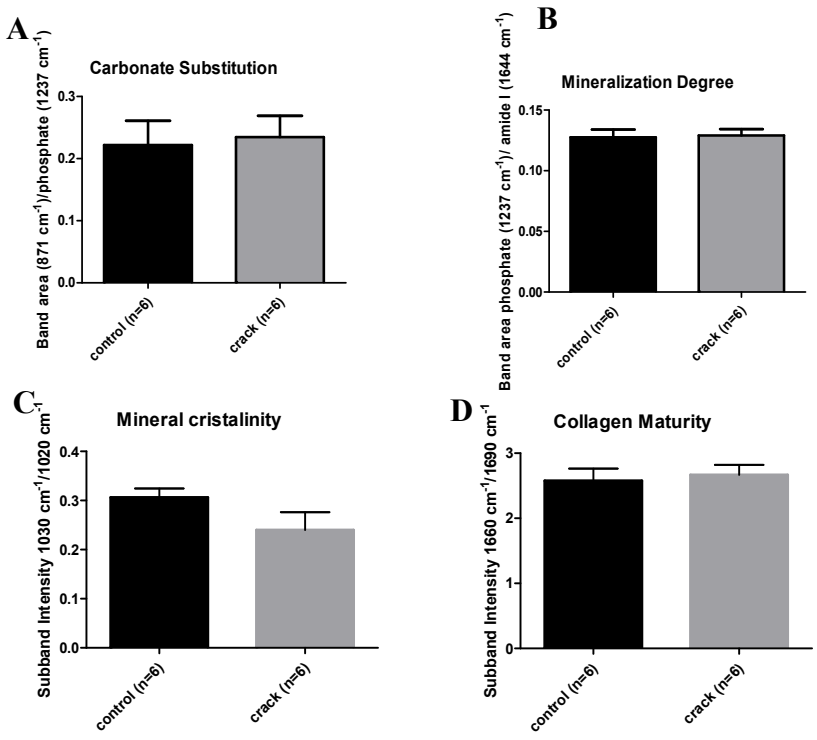

Figure 3. FTIR parameters results. Parameters evaluated in femur of Control (n=6) and Crack rats (n = 6). **A.** Mean ± SEM of Carbonate Substitution analyzed by t-Student parametric test ($p > 0.05$). **B.** Mean ± SEM of Mineralization Degree analyzed by t-Student parametric test ($p > 0.05$). **C.** Mean ± SEM of Mineral Crystallinity analyzed by t-Student parametric test ($p > 0.05$). **D.** Mean ± SEM of Collagen Maturity analyzed by t-Student parametric test ($p > 0.05$).

Table 1. Components identified according to peak frequency and vibrational mode

Peak Frequency (cm^{-1})	**Component Identification**
1644	**Amide I (YANG *et al.*, 2005))**
1537	**Not identified**

1451	**Not identified**
1402	**CH3 Symetric deformation (AGARWAL, *et al.*, 2006))**
1327	**Phosphate I (DOVBESHKO, *et al.*, 1997)**
1030	**Collagen (FUJIOKA *et al.*, 2004)**
871	**Carbonate (AGUIAR, 2012)**

3.3. Micro-CT evaluation

The representative 3D reconstructed micro-CT images of the femur (cortical, femural head and epiphyseal disk) from control and crack rats are shown in Figure 4 (A-F. Bone parameters were evaluated for cortical region and trabecular region (femoral head and epiphyseal disk). There were no difference ($p > 0.05$) in bone volume percentage degree of anisotropy in control and crack rats (Figure 5. A-B). However, fractal dimension was increase ($p < 0.05$) in crack exposed compared with control rats (Figure 5C). Besides, the the number of closed pores was strongly increased ($p < 0.05$). in crack exposed than control rats. The parameters for trabecular evaluation, both in the femoral head region and in the epiphyseal disk: a percentage of bone volume; degree of anisotropy; trabecular thickness; trabecular number; trabecular separation; no differences were observed between crack and control rats ($p > 0.05$) (Figure 6A-J).

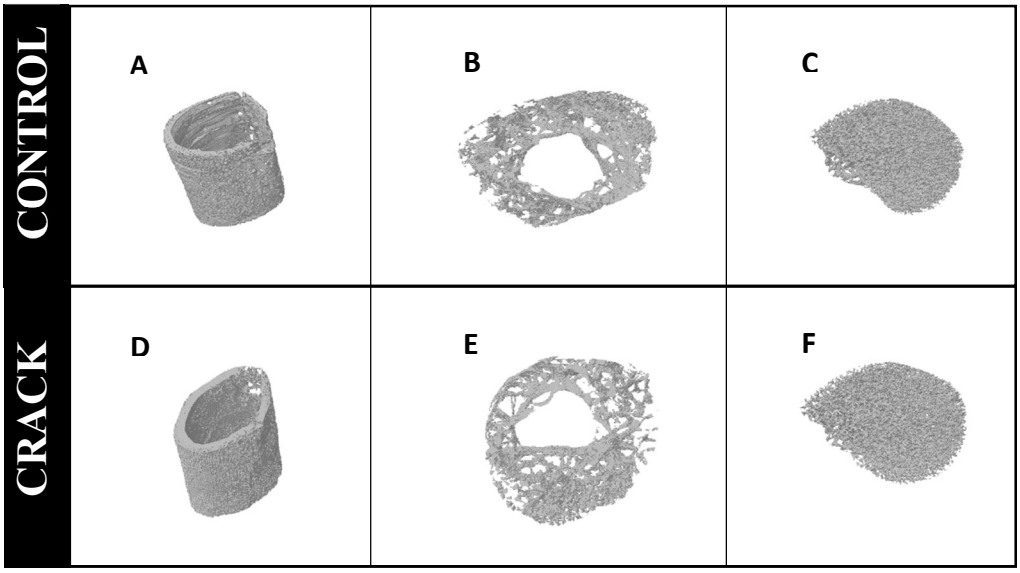

Figure 4. Micro-CT 3D rendering. Three-dimensional images of the regions of femur rats control and crack. **A.** Image of cortical region of control rats. **B.** Image of trabecular region of the epiphyseal disk of control rats. **C.** Image of trabecular region of femur head. **D.** Image of cortical region of Crack rats. **E.** Image of trabecular region of the epiphyseal disk of Crack rats. **F.** Image of trabecular region of femur head of Crack rats.

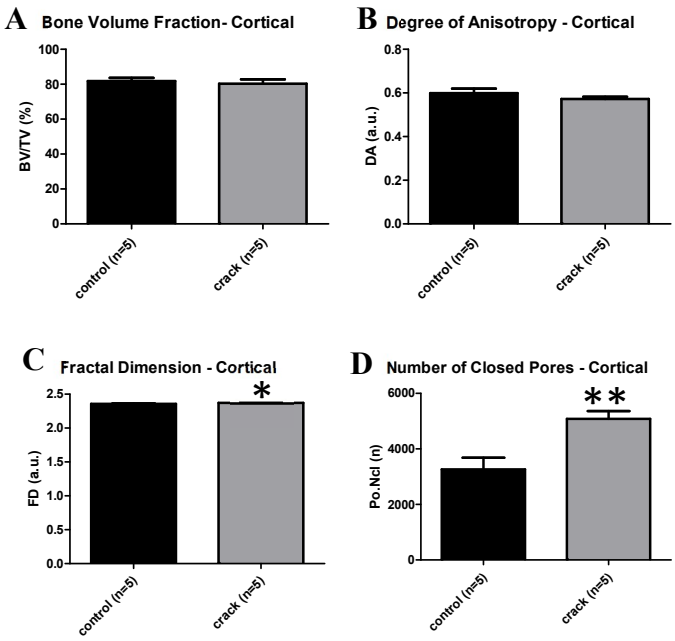

Figure 5. Micro-CT parameters cortical bone. Bone Quality Parameters evaluated in the Micro-CT in region of femurs of control (n=5) and crack rats (n = 5). **A.** Mean ± SEM of Bone Volume Fraction (%) analyzed by the t-Student parametric test ($p > 0.05$). **B.** Mean ± SEM of Degree of Anisotropy (a.u.) analyzed by the non-parametric Mann-Whitney test ($p > 0.05$). **C.** Mean ± SEM of Fractal Dimension (a.u.) analyzed by the t-Student parametric test ($*p < 0.05$). **D.** Mean ± SEM of Number of Closed Pores (n) analyzed by the t-Student parametric test ($**p < 0.05$).

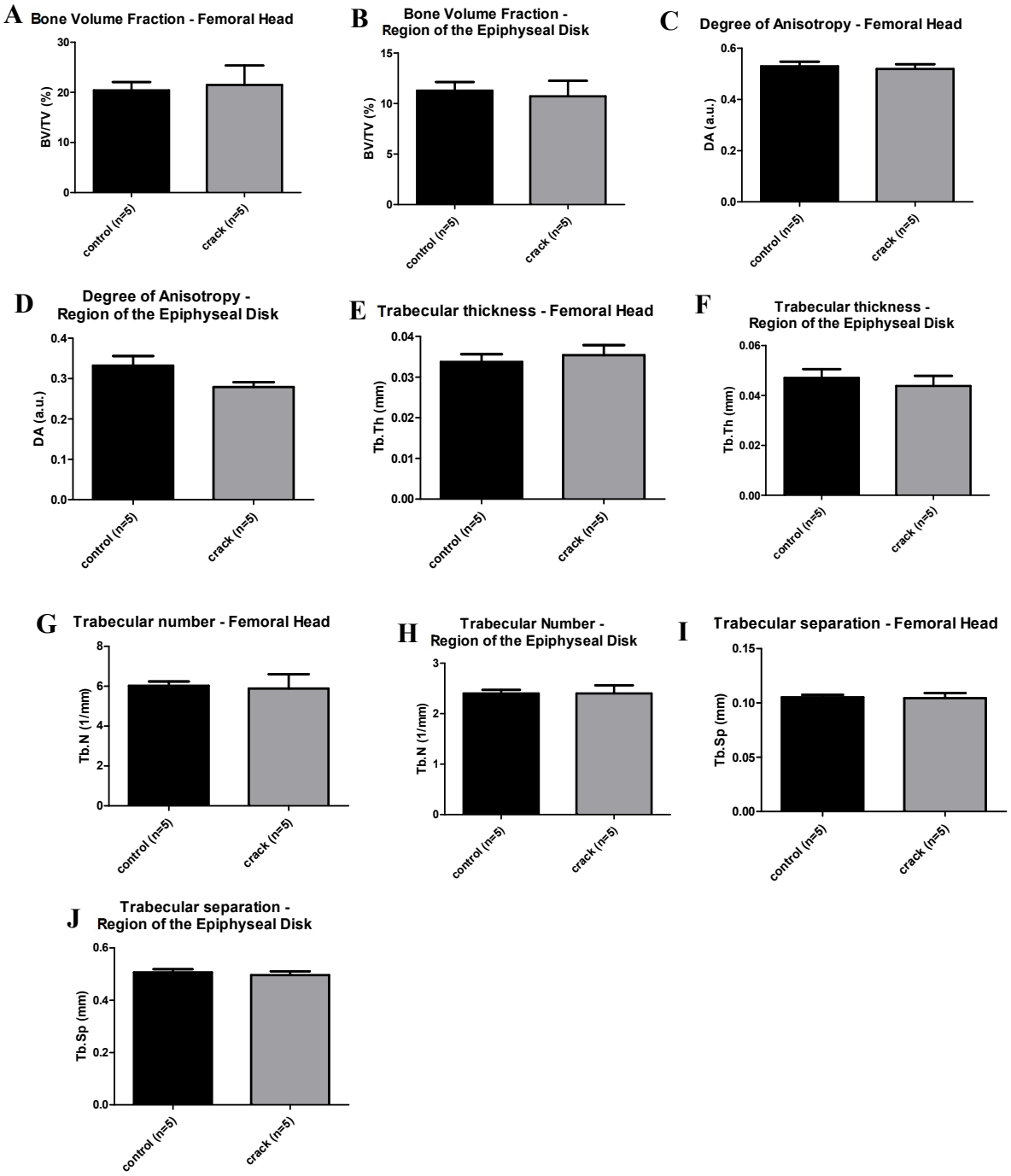

Figure 6. Micro-CT parameters trabecular bone. Bone Quality Parameters evaluated in the Micro-CT in region trabecular (femoral head and epiphyseal disk) of femurs of control (n=5) and crack rats (n = 5). **A.** Mean ± SEM of Bone Volume Fraction of femoral head (%) analyzed by the analyzed by the t-Student parametric test ($p > 0.05$). **B.** Mean ± SEM of Bone Volume Fraction of region of the epiphyseal disk (%) analyzed by the t-Student parametric test ($p > 0.05$). **C.** Mean ± SEM of

Degree of Anisotropy of femoral head (%) analyzed by the analyzed by the t-Student parametric test ($p > 0.05$). **D.** Mean ± SEM of Degree of Anisotropy of region of the epiphyseal disk (%) analyzed by the t-Student parametric test ($p > 0.05$). **E.** Mean ± SEM of Trabecular thickness of femoral head (mm) analyzed by the analyzed by the t-Student parametric test ($p > 0.05$). **F.** Mean ± SEM of Trabecular thickness of region of the epiphyseal disk (mm) analyzed by the t-Student parametric test ($p > 0.05$). **G.** Mean ± SEM of Trabecular number of femoral head (1/mm) analyzed by the analyzed by the t-Student parametric test ($p > 0.05$). **H.** Mean ± SEM of Trabecular number of region of the epiphyseal disk (1/mm) analyzed by the t-Student parametric test ($p > 0.05$). **I.** Mean ± SEM of Trabecular separation of femoral head (mm) analyzed by the analyzed by the t-Student parametric test ($p > 0.05$). **J.** Mean ± SEM of Trabecular separation of region of the epiphyseal disk (mm) analyzed by the t-Student parametric test ($p > 0.05$).

4. Discussion

This study demonstrated that the crack used during the gestational period was able to alter the bone profile of the rats that were exposed during the intrauterine phase in the bone microarchitecture and mineral composition of the bone.

Among the determinants of bone strength are mineral properties, including carbonate substituion, degree of mineralization, mineral crystallinity and collagen maturity; the understanding of bone mineral complexity is important in determining bone quality and the mechanisms that trigger bone fragility (FARLAY and BOIVIN, 2012). Among these determinants analyzed no significant difference was found between group contreol and crack group

In the FTIR analysis, only the 1402 cm^{-1} was significant, being increased in crack rats, but the vibrational mode of this peak represents a symmetric deformation of CH3, and at this wavelength vibrates CH3 symmetric deformation and Symmetric CH3 bending modes of the methyl groups of proteins.

Bone quality can be measured through changes in bone microarchitecture (CHAPPARD *et al.,* 2008). In the present study, we evaluated the microarquity of the femur through the MictoCT. The parameters analyzed in MicroCT are in accordance with the recommendations of the American Society for Bone and Mineral Research. In

the trabecular bone, no alterations were found in the crack rats; in the cortical bone (femoral diaphysis) an increase in the fractal dimension (FD) and the number of closed pores (P.oNcl) in the rats were observed. Exposed to crack in the gestational period, when compared to the control rats; demonstrating that the crack used during pregnancy promoted alteration in the cortical bone microarchitecture of crack rats.

The FD measures the complexity of the structural pattern, expressed in roughness and texture (CHAPPARD *et al.,* 2008). Studies have shown that the increase of FD is related to a better bone quality, since the decrease of this parameter is observed in bone pathologies that damage the microarquiterura of the bone, expressed by the bone resorption (KOH, PARK and KIM, 2012; ARAÚJO, 2013).

An increased value of P.oNcl appears to be related to improved bone quality, as was observed in a study evaluating a drug for treatment of bone resorption pathology (osteoporosis). Through which it was possible to observe that the animals induced to have this disease presented a lower P.oNcl than the animals of the control group and those of the group treated with the drug, that is, the increase of P.oNcl of the group treated with the drug would be promoting an improvement in bone quality (YOGUI, *et al.,* 2018); another given that corroborates with our research is that in this study there was also no significant difference in bone volume between the groups. In another study evaluating bone pathology, we observed a decrease in cortical porosity in diseased infants (BOSKEY et al., 2002).

The increased value of FD and P.oNcl suggest that there was an improvement in bone quality, another research that corroborates with our data is that a reduction in P.oNcl associated with a reduction of FD indicated a deterioration of the bone structure (SANTOS, 2002), confirming that the inverse result would be indicative of an improved bone. The literature clearly describes the deleterious effects that use during pregnancy impact to the fetus (CAMPILO *et al.,* 2004; MATOS et al., 2011; MILLIAM and JOHNS, 2014); however there are no reports on bone quality, it was expected in our research that there would be a compromise in the microarquity of the analyzed femurs, as well as in the molecular components. However, our results demonstrate the opposite, because there was an improvement in the bone microarquitetura, measured by the increase of FD and P.oNcl; as well as an improvement in bone quality, in relation to stiffness, due to the increase of mineral crystallinity. We do not believe that crack promotes an improvement in bone quality, what we can suggest is a defense mechanism

occurred during the bone formation of rats exposed to crack smoke, and the bone tissue acted as a protection factor against these metabolites.

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APÊNDICE

Biomechanical test

For this analysis the left femurs were used, which were thawed twenty-four hours prior to the test at -4c, and one hour before the test were placed at room temperature involved by gauze soaked in PBS until the start of the experiment. Each left femur was submitted to a three-point flexion test until fracture using a material testing machine (EMIC DL 2000, EMIC Equipamentos e Sistemas de Ensaio Ltda, São José dos Pinhais, Brazil). Each specimen was positioned horizontally in two support attachments with a 10 mm span between them, the femoral head positioned upwards; and a 50 kg load was applied to the central portion of the femur, which portion was measured and established prior to the test. The load and displacement data were recorded and the load versus displacement curves were plotted. The maximum load values were obtained from the data strength (N), energy for fracture (mJ) and stiffness values (N / mm) were calculated as the slope of the initial linear loading portion of the curves.

Results - Biomechanical testing

The 3-point bending test produces mid-diaphysis fractures under controlled loading conditions. The primary analysis of the test measures 3 elements of biomechanical performance: force (load, or the force applied to the bone), energy to fracture (the area under the load-displacement curve) and stiffness. Crack had no effect ($p > 0.05$) in force, energy to fracture and stiffness than control rats (Figure 1. A-C).

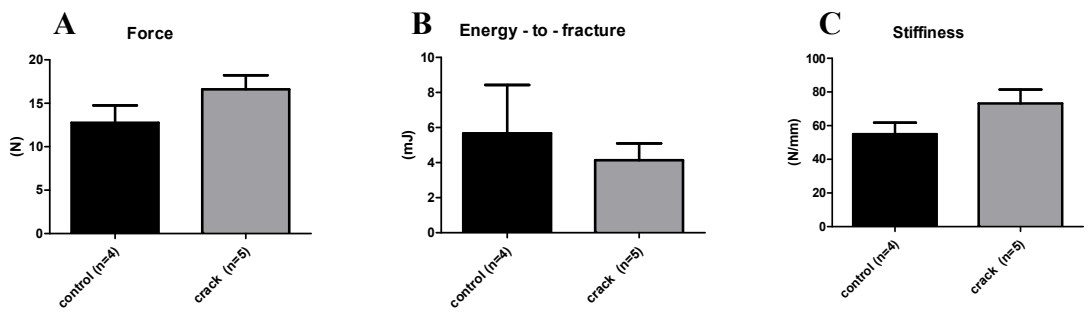

Figure 1. Biomechanical testing. Biomechanical parameters evaluated in the 3-point flexion test performed on femur of control (n=4) and crack rats (n = 5) and analyzed by the tStudent parametric test ($p < 0.05$). **A.** Mean ± SEM of maximum force reached until fracture (Force, N); **B.** Mean ± SEM of energy spent to fracture the bone (Energy-tofracture, mJ); **C.** Mean ± SEM of stiffness offered by bone during force application (stiffness, N/mm).