Pontifícia Universidade Católica do Rio Grande do Sul

Faculdade de Biociências

Programa de Pós-Graduação em Biologia Celular e Molecular

Estudo das Alterações Mitocondriais Provocadas na Sepse por Espectroscopia de Infravermelho pela Transformada de Fourier e Refletância Total Atenuada

Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular como requisito parcial para a obtenção do grau de Mestre.

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Porto Alegre

Outubro de 2006

AGRADECIMENTOS

Ao Laboratório de Química de Produtos Naturais da Faculdade de Química e ao Laboratório de Biofísica da Faculdade de Biociências da PUCRS.

Em especial, ao meu orientador, André Arigony Souto, pela paciência e compreensão, pelas risadas e lições que vou levar para a vida.

Ao professor Jarbas Oliveira pelo estímulo e pela confiança no trabalho que estava sendo feito.

A diretora da Faculdade de Farmácia Flávia Valladão Thiesen pela oportunidade de estagiar ao seu lado e pelo apoio profissional.

Aos demais professores e funcionários da Faculdade de Química e do Instituto de Biociências da PUCRS.

Aos meus pais queridos, Oscar e Regina; irmãos, Luiza e Carlos; avós, Isolde, Noé e Lahir; tios, Gladys, Paulo, Ana, Eduardo, Leco, Henrique e cônjuges; e primos, Lucas, Gabriele, Fernando, Fabi, Flavia, Mariana, Bruno e Fred, que torcem por mim, o meu muito obrigado por tudo. Vocês são meu alicerce. Amo muito todos vocês.

Ao meu amigo, Leonardo Marchiori, pois sem sua ajuda e incentivo não teria como realizar este sonho.

Aos meus amigos, Carolina Bastos e Paulo Ramos, que me ajudaram na concretização desse projeto, incluindo as tardes de sábado no laboratório e que não me deixaram desanimar. Meus mais sinceros agradecimentos, pois mais que colegas de trabalho, são meus amigos.

A todos aqueles que me ajudaram nas pequenas coisas, seja com palavras amigas ou no momento em que precisei de uma mãozinha: Siomara , Paulão, Matias Frizzo, Ju - a Fredo, Márcia Kober e Fabio – vocês são demais – Ju Torres, Josiane Bortoloto, Adroaldo Lunardeli, Márcia Pimentinha, Zé Mateus, Carlos, Cati, Rodrigo (Toco), Vasyl, Bento, Mel, Fernanda, Eduardo, Luciana. Aos meus novos amigos, Michele, Mauro e Giancarlo pelas risadas. A todos aqueles que se fizeram presentes em minha vida.

ÍNDICE

RELAÇÃO DE FIGURAS

REVISÃO BIBLIOGRÁFICA

ARTIGO

LISTA DE ABREVIATURAS

ADP	**Adenosina Difosfato**
ATP	**Adenosina Trifosfato**
ATR	**Attenuated Total Refletance**
CK	**Creatina Quinase**
CKMB	**Creatina Quinase fração MB**
ERO	**Espécie Reativa de Oxigênio**
FTIR	**Fourier Transformed Infrared**
IRF	**Índice de Refração**
IV	**Infravermelho**
LLC	**Leucemia Linfocítica Crônica**
LPS	**Lipopolissacarídeo**
NO	**Óxido Nítrico**
P-gp	**Glicoproteína P**
SIRS	**Síndrome da Reação Inflamatória Sistêmica**
TOM	**Translocase Outer Membrane**
UTI	**Unidade de Tratamento Intensivo**
ZnSe	**Selenito de Zinco**
BaF_2	**Fluoreto de Bário**
CaF_2	**Fluoreto de Cálcio**
KBr	**Brometo de Potássio**
KCl	**Cloreto de Potássio**

RESUMO

A espectroscopia de infravermelho (IV) permite caracterizar molecularmente tecidos e fluídos, uma vez que seus espectros são característicos e atuam como uma impressão digital. O rápido desenvolvimento da técnica de infravermelho na última década tem aberto novas portas para o prognóstico e diagnóstico de patologias facilitando a procura de melhores tratamentos. Atualmente, o infravermelho (IV) vem sendo utilizado nos mais diversos modelos experimentais de sistemas biológicos. A sepse provoca mudanças no ambiente celular e em suas trocas iônicas, afetando o funcionamento da mitocôndria e aumentando a formação de espécies reativas de oxigênio (EROs). O óxido nítrico (NO) é considerado um marcador da formação de radicais livres produzidos, enzimaticamente ou não, nos tecidos. O aumento dos radicais livres promove a oxidação de resíduos de aminoácidos, aumentando a formação de grupos carbonílicos (CO) protéicos que atuam como biomarcadores do estresse oxidativo. Devido a esses fatores, entre outros, as mitocôndrias e seus complexos vêm sendo avaliados pela espectroscopia de infravermelho nas regiões das bandas de amidas I (espectro entre 1615 cm $^{-1}$ e 1700 cm $^{-1}$, referente a ligações C=O) e II (espectro entre 1500 cm $^{-1}$ e 1600 cm $^{-1}$, referente a ligações C-H), característico de proteínas, com o objetivo de observar alterações que possam ocorrer nestas organelas.

No presente trabalho de mestrado, nosso objetivo principal foi analisar as alterações em mitocôndrias hepáticas causadas pela sepse induzida por CLP em ratos (*Rattus novergicus* – var. Wistar) através do FTIR-ATR. O experimento foi contemplado pela extração das mitocôndrias hepáticas através de diferentes gradientes de centrifugação em meios contendo sacarose, heppes e EGTA. A dosagem de NO sérico foi feita através da metodologia de Griess. Nossos resultados apresentaram diferenças significativas nas bandas de amida I do espectro de infravermelho do grupo séptico em relação ao controle, controle anestesia e sham. As mesmas diferenças foram percebidas para a dosagem de NO sérico.

APRESENTAÇÃO DO TEMA

Introdução

O infravermelho tem sido utilizado nas últimas décadas em diversas áreas do conhecimento. Atualmente, sua utilização está direcionada para o uso prognóstico e diagnóstico de patologias e em modelos experimentais de sistemas biológicos (LIU KZ, SHI MH, MANTSCH HH 2005; NAUMANN D 2000; SHAW RA, MANTSCH HH 2000).

A sepse, inflamação sistêmica em conseqüência de uma infecção, tem sido alvo de muitos estudos, pois é responsável por um grande número de óbitos nas UTIs hospitalares. É caracterizada por diversas alterações metabólicas e teciduais, afetando a estrutura da membrana mitocondrial e, conseqüentemente, a permeabilidade e o fluxo de íons (NUNES FB et al 2003; CHEN HW et al 2004; KIM JS et al 2004).

A seguir, apresentaremos os fundamentos da espectroscopia de infravermelho e suas aplicações em sistemas biológicos. Também será abordado o processo de sepse, suas alterações em mitocôndrias hepáticas e estudos correlacionando o infravermelho, a mitocôndria e seus complexos.

Princípios do infravermelho

O infravermelho encontra-se na faixa do espectro não visível (entre 780 nm/12820 cm^{-1} e 1 nm/10cm^{-1}) e é dividido em próximo, médio e distante (SHAW A 2000). A espectroscopia de infravermelho (IV) mede a freqüência e a intensidade na qual uma dada amostra absorve a radiação infravermelha. Assim, o espectro de infravermelho representa através de picos de absorção, a freqüência de vibração dos átomos constituintes do material, identificando os componentes químicos da amostra. A intensidade de absorção da amostra relaciona-se com a concentração de um respectivo componente, possibilitando uma análise quantitativa.

Basicamente, as vibrações moleculares são classificadas em dois tipos: vibrações de deformação axial (*stretching*) e de deformação angular (*bending*). As deformações axiais, ou estiramento são oscilações radiais das distâncias entre os núcleos, enquanto as deformações angulares envolvem mudanças dos ângulos entre as ligações ou, como no modo de deformação assimétrica fora do plano, alterações do ângulo entre o plano que contém as ligações e um plano de referência (FREEMAN EC, PAUL W 1978).

Os grupamentos funcionais de compostos orgânicos absorvem em freqüências características no infravermelho. Assim, em um gráfico de intensidade de radiação versus freqüência, o espectrograma de infravermelho permite caracterizar os grupos funcionais de um padrão ou de um material desconhecido. As posições das bandas no espectro de infravermelho são apresentadas em números de ondas, cuja unidade é o centímetro inverso (cm^{-1}) e as intensidades das bandas são vistas como absorbância (SILVERSTEIN RM 1994).

No Infravermelho por Transformada de Fourier (FTIR), a radiação, contendo todos os comprimentos de onda, depois de colimada por um espelho, é introduzida em um interferômetro de Michelson (dispositivo formado por um divisor de feixe) e separada em dois feixes, um deles percorrendo uma distância fixa e o outro, uma distância variável (espelho móvel) (Figura 1). No divisor de feixe, os dois raios são combinados opticamente, podendo gerar uma interferência construtiva (se estiverem em fase) ou destrutiva (se estiverem fora de fase). Quando este feixe combinado de luz atravessa a amostra, é absorvido seletivamente e, dependendo das absorções apresentadas pela amostra, gera um interferograma. Este interferograma pode ser tratado por meio de um processo matemático, denominado transformada de Fourier, originando um espectro ou padrão de absorção da amostra, ou seja, seu espectro no infravermelho, que pode ser tanto de transmitância quanto de absorbância (WARTEWIG S, REINHARD HH, NEUBERT T 2005). Essa técnica permite a aquisição de centenas de espectros de infravermelho em apenas alguns minutos. Os espectros isolados são combinados no computador, originando um espectro no qual os ruídos de fundo do equipamento podem ser bastante reduzidos, produzindo, portanto, um espectro limpo.

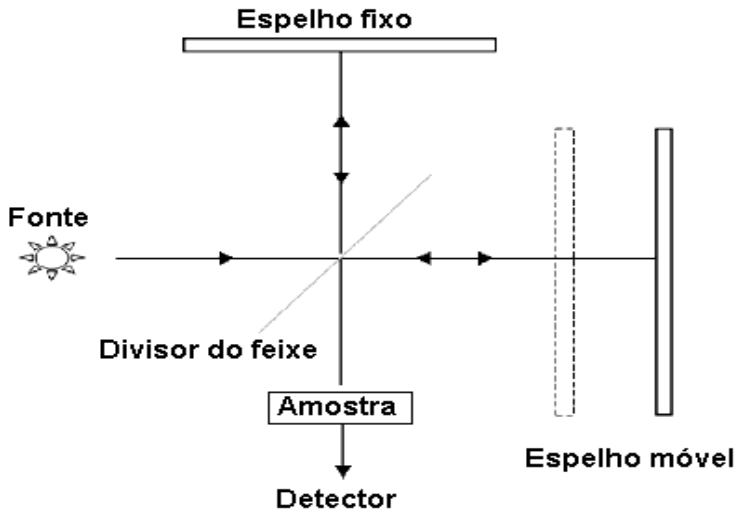

Figura 1: Interferômetro de Michelson. Representação esquemática adaptada de Wartewig et al2005

O uso de Atenuação de Refletância (ATR) em espectroscopia baseia-se no fato de que, embora ocorra completa reflexão interna na interface cristal/amostra, a radiação penetra de fato uma pequena distância dentro da amostra. Esta penetração é chamada de onda evanescente. A amostra interage com a onda evanescente, resultando na absorção da radiação pela amostra, que corresponde ao espectro de transmissão dessa mesma amostra, conforme a representação esquemática da refletância total exibida na Figura 2 (WARTEWIG S, REINHARD HH, NEUBERT T 2005; LUCASSEN GW, VEEN GNA, JANSEN JAJ 1998).

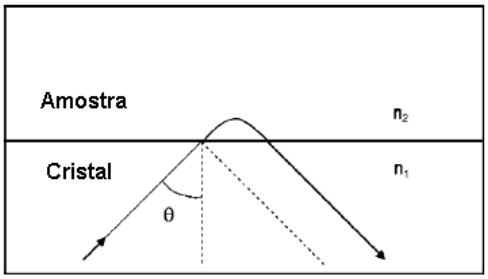

Figura 2: Refletância total atenuada. Esquema adaptado de Wartewig et al 2005

O espectro depende de diversos parâmetros, incluindo ângulo de incidência da radiação na amostra, comprimento de onda da radiação, índices de refração da amostra e do cristal (cristais de ZnSe, BaF2, CaF2, KBr, KCl) do equipamento (WARTEWIG S, REINHARD HH, NEUBERT T 2005). A profundidade da penetração, d_p, da onda evanescente, definida como a distância requerida para a amplitude do campo elétrico caia para $1/e$ de seu valor na interface é dada por:

$$d_p = \frac{\lambda}{2\pi n_1 \sqrt{\sin^2\theta - (n_2/n_1)^2}}$$

Onde:

λ= comprimento de onda da radiação incidente

n_1 = índice de refração do cristal (IRF)

n_2 = IRF da amostra

θ = ângulo de incidência

Ou seja, a profundidade de penetração depende do comprimento de onda da radiação (WARTEWIG S, REINHARD HH, NEUBERT T 2005; LUCASSEN GW, VEEN GNA, JANSEN JAJ 1998).

As vantagens em utilizar a técnica de infravermelho estão na facilidade do manuseio, a não utilização de reagentes e a determinação de diferentes analitos em um único espectro (LILY MN et al 1999; SHAW A et al 2000). O material a ser analisado pode ser líquido, gás, filme seco, em pó e *in natura*. Sendo assim, é possível analisar alterações teciduais no organismo intacto, o que torna a espectroscopia de infravermelho uma técnica não invasiva, dependendo do órgão ou material a ser analisado (LILY MN et al 1999).

Sistemas Biológicos

Em 1911, W.W. Coblentz foi o primeiro cientista a propor que poderia se obter informação de materiais biológicos através da espectroscopia de infravermelho. Essa técnica foi utilizada para diferenciar e identificar bactérias nas décadas de 1950 e 1960. Novos resultados publicados em 1959 definiram que o infravermelho não poderia ser utilizado em um esquema prático, pois naquela época as especificações da técnica, como a sensibilidade, o tempo e a reprodutibilidade, eram limitadas. (RIDDLE et al 1956; NORRIS 1959; MURRAY et al 1999). Este panorama foi modificado nas décadas de 80 e 90 pela superação destes problemas técnicos.

Desde então, o infravermelho vem sendo utilizado para análise vibracional de moléculas de complexos biológicos (JACKSON M, MANTSCH HH 1996), apresentando resultados significativamente correlacionados quando comparados a técnicas padrão já existentes. Nas células e fluídos, a maior absorção espectral está situada nas bandas referentes às ligações N-H, C=O, C-O, C-H e P=O encontradas em proteínas, lipídeos, ácidos nucléicos, carboidratos e açúcares (Figura 3).

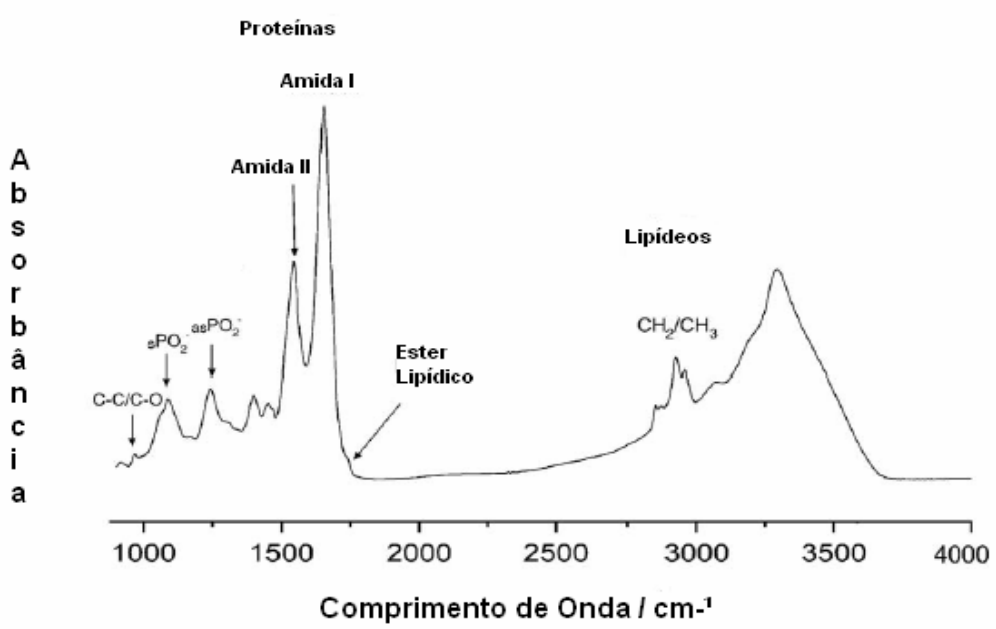

Figura 3: Espectro adaptado e representativo do infravermelho de material biológico no intervalo de 1000 cm-¹ a 4000 cm-¹ (LIU KZ, SHI MH, MANTSCH HH 2005).

Diagnóstico Clínico

Na área da hematologia, o IV pode auxiliar no prognóstico e no diagnóstico de leucemias e da ß - talassemia, possibilitando identificar, através de diferenças em seus espectros, se há sensibilidade ou resistência aos quimioterápicos, além de avaliar o processo de apoptose nas células sangüíneas (LIU KZ, SHI MH, MANTSCH HH 2005).

SCHULTZ et al mostram em seus estudos, a heterogeneidade dos espectros de infravermelho de células normais e leucêmicas. A banda representada pelo número 1, originada de ligações C–C/C–O, envolve características de desoxirribose e grupos fosfato de parte do DNA. As bandas representadas pelos números 2 (1087 cm^{-1}) e 3 (1240 cm^{-1}) são originadas, respectivamente, de ligações simétricas e assimétricas do estiramento das vibrações de íons dos grupos fosfato (PO_{2-}) que fazem parte do DNA. A análise estatística, baseada em *clusters* hierárquicos separou as células leucocitárias normais das células de Leucemia Linfocítica Crônica (LLC) (Figura 4). As células normais foram classificadas em dois subgrupos e as células de LLC foram divididas em três subgrupos (SCHULTZ CP, LIU KZ, JOHNSTON JB et al 1996). Claramente, o espectro de infravermelho contém informação suficiente para diferenciar as células, podendo ser utilizado no prognóstico e no diagnóstico.

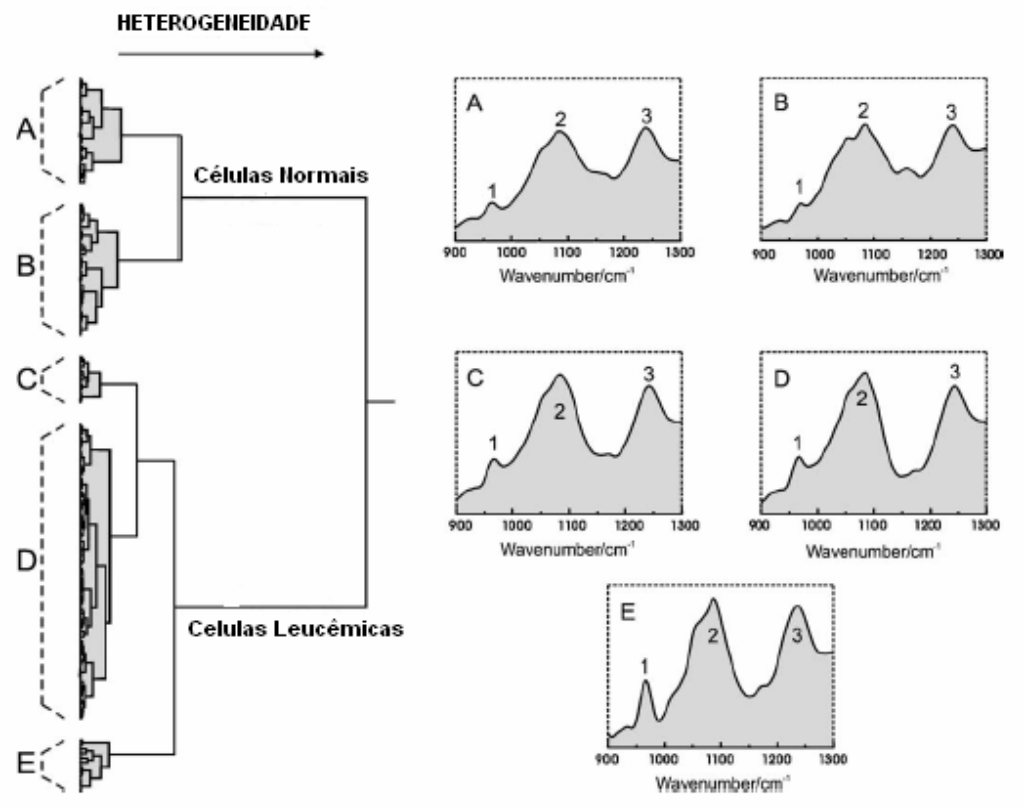

Figura 4: Representação de espectros de infravermelho de linfócitos normais (A e B) e células de LLC (C,D e E) separados por clusters (SCHULTZ CP, LIU KZ, JOHNSTON JB et al 1996)

A resistência a múltiplas drogas ocorre freqüentemente nos tratamentos antineoplásicos devido a superexpressão de proteínas de membrana, chamadas glicoproteínas p (P-gp) e outras mudanças moleculares nas frações lipídicas e de ácidos nucléicos das células. Na última década, o infravermelho vem sendo empregado no entendimento das modificações bioquímicas que ocorrem na resistência a múltiplas drogas e tem se caracterizado pelas diferenças nas bandas de amida I e II, sendo capaz de diferenciar as linhagens de células leucêmicas sensíveis das resistentes. Além dessas alterações nas bandas referentes às proteínas, o infravermelho captou o decréscimo nas faixas espectrais dos lipídeos e dos ácidos nucléicos (LIU KZ, SHI MH, MANTSCH HH 2005).

A talassemia compreende um grupo de desordens genéticas caracterizadas por alteração na síntese de hemoglobina, causada por mutações que diminuem ou eliminam a síntese de α ou β globina que fazem parte da hemoglobina. Esses grupos mutados podem ser diferenciados da hemoglobina normal pela espectroscopia de infravermelho. Uma surpreendente mudança foi observada na banda de ligação assimétrica de PO_2-. A banda em 1260 cm^{-1} foi observada na membrana de eritrócitos normais com uma banda adicional em 1230 cm^{-1}, possivelmente pela presença de grupos esteróis. Ao contrário, a banda de ligações PO_2- dos eritrócitos alterados aparece em 1230 cm^{-1} com a ausência da banda de 1260 cm^{-1}, mostrando que a oxidação dos grupos esteróis das hemácias modifica a posição das bandas no espectro de infravermelho (LIU KZ, SHI MH, MANTSCH HH 2005; LIU KZ, TSANG KS, LI CK et al 2003).

A apoptose, um processo ativo que envolve mudanças bioquímicas em três componentes celulares essenciais: DNA, proteína e lipídeo (LIU KZ, MANTSCH HH et al, 2001), esses vêm sendo estudados com o objetivo de classificar o estágio em que se encontra determinada doença. Células humanas leucêmicas HL60 (GASPARRI F, MUZIO M 2003) foram incubadas com captotecina, uma droga citotóxica, sendo monitorados os tempos em que essas células entravam em apoptose. Fortes mudanças no espectro de infravermelho foram detectadas durante o processo, sugerindo que no futuro o ATR-FTIR poderá ser utilizado no monitoramento e na distinção de células apoptóticas, podendo analisar o efeito de drogas anticancerígenas e da radioterapia no combate às doenças malignas.

A xerose senil (pele seca) é muito comum em pessoas idosas, causando desconforto e desordens dermatológicas. Nosso grupo desenvolveu, em dissertação de mestrado (MILAN 2006) e tese de doutorado (CORTE 2006), o uso de emulsões cosméticas para prevenir e tratar a pele seca, sendo analisada a hidratação da pele pela espectroscopia de infravermelho nas bandas de amida I e II, mostrando mais uma vez a ampla utilidade dessa técnica.

Modelos Experimentais

A citologia convencional envolve colorações de células e observação ao microscópio, procurando diferenciar células alteradas de células normais e dependendo do conhecimento e da

interpretação do observador, podendo resultar em diagnósticos equivocados. Além disso, essa técnica não é capaz de determinar modificações celulares antes que sua morfologia e seu aspecto estejam alterados. Baseados nessa premissa, pesquisadores induziram câncer em células epiteliais de camundongos e compararam o método citopatológico convencional com os resultados obtidos pelo infravermelho do DNA dessas células (Figura 5). Eles chegaram à conclusão de que é possível distinguir alterações nessas células antes mesmo de o tumor ser detectado visualmente pela análise histológica ou se tornar palpável (MALINS DC et al 2004, MORDECHAI S et al 2004, MOURANT JR et al 2003).

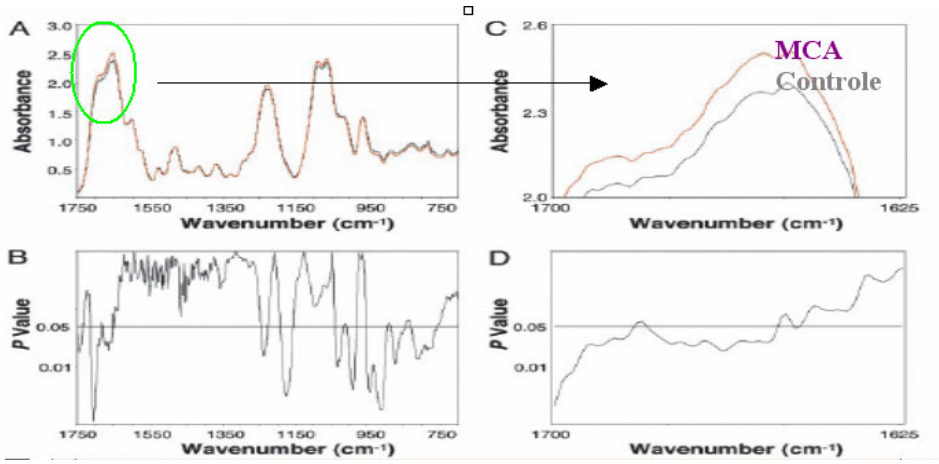

Figura 5: Espectro do DNA de células epiteliais no intervalo de 1750 a 760 cm-¹ (A), mostrando diferenças significativas (valores de *p< 0,05* em B e D) entre o grupo controle e o grupo no qual foi induzido o câncer com MCA (3-metilclorantreno). Em C ampliação da faixa espectral de 1700 a 1625 cm-¹ (MALINS DC et al 2004)

A vantagem do FTIR está em facilitar e examinar as regiões restritas de tecidos e culturas de células. Ensaios feitos com essa tecnologia foram sensíveis e efetivos para a detecção de células infectadas por membros das famílias dos herpes vírus e dos retrovírus. Diferenças detectáveis e significativas no espectro entre células normais e infectadas foram evidentes em estágios iniciais da infecção. Mudanças significativas nos parâmetros espectroscópicos foram observadas entre as células infectadas e as não infectadas, estando diretamente correlacionadas com as mudanças no comportamento específico do vírus infectante (ERUKHIMOVITCH et al 2005). Em outro estudo, estruturas do tecido nervoso de hamsters infectados por prions foram analisadas pela microespectroscopia de infravermelho convencional juntamente com a luz de sincrotron. Células neurais foram separados da raiz do gânglio dorsal e escaneados, sendo possível analisar *in situ,* no interior das células isoladas, as diferenças estruturais das proteínas dos *hamsters* infectados e dos não infectados. Essas células exibiram áreas aumentadas contendo estruturas folha β-pregueada, as quais foram co-localizadas constantemente com acúmulo de proteínas priônicas (PrP^{Sc}). Dados espectrais foram obtidos por purificação de PrP^{Sc} isolados de tecidos nervosos de hamsters

infectados para elucidar similaridades/dissimilaridades entre as estruturas *in situ* e *ex vivo* (KNEIPP J et al 2002; KNEIPP J, MILLER LM, SPASSOV S et al 2004; NAUMANN D et al 2002; BRIAN JB, VALERIE D 2002).

Sepse

A inflamação é uma resposta orgânica local ou sistêmica, de magnitude variável, desencadeada por diversos fatores, com o objetivo de proteger-nos contra qualquer agressão, regulando e mantendo o equilíbrio das funções corporais. No entanto, essa resposta muitas vezes é direcionada de forma destrutiva, rompendo a harmonia e o equilíbrio químico do organismo.

Essa resposta natural orgânica geralmente é percebida através das alterações cardiovasculares (taquicardia, aumento da contratilidade e débito cardíaco), respiratórias (taquipnéia), neuro-endócrinas (catecolaminas, cortisol, hormônio antidiurético, hormônio do crescimento, glucagon e insulina), imunológicas (fator de necrose tumoral, interleucinas, interferon, polimorfonucleares), entre outras (KIRKEBOEN KA, STRAND AO 1999). Há, também, a ativação dos leucócitos, aumento de sua agregação na microcirculação, maior infiltração celular numa verdadeira explosão respiratória para a produção de substrato energético e o conseqüente aumento no consumo de oxigênio. Bone (1992) descreve a Síndrome da Reação Inflamatória Sistêmica (SIRS) em três estágios: I) resposta inflamatória local; II) resposta inflamatória sistêmica controlada - nessas duas primeiras fases temos as reações protetoras e promotoras que evoluem até a reparação da lesão, o combate da infecção e a recuperação da homeostase; e III) situação em que a homeostase não se recupera, há uma reação sistêmica maciça onde os efeitos dos mediadores são predominantemente destrutivos. Reações catastróficas são desencadeadas pela ativação do sistema retículo-endotelial, onde há perda da integridade das membranas separadoras dos compartimentos corporais, com a lesão de vários órgãos. Há também uma vasodilatação descontrolada e sistêmica, e a conseqüente diminuição da resistência periférica, necessária para a demanda funcional do sistema cardiovascular e o direcionamento para a economia energética (BALK RA, BONE RC 1989; BONE RC et al 1992; CASEY L, BALK RA, BONE RC 1993), podendo levar à falência da microcirculação e à disfunção múltipla de órgãos (fígado, rim, pulmão e coração).

O termo sepse (do grego *sêpsis*) significa "putrefação". Responsável por um grande número de falecimentos (20 a 60%) nas Unidades de Tratamento Intensivo (UTIs), em decorrência de uma inflamação sistêmica como resposta à infecção, pode evoluir de um simples contágio à severidade, e desta ao choque séptico (BREALEY D et al 2002).

Sabe-se que bactérias gram negativas são as principais causadoras desta síndrome e que elas, assim como seus produtos bacterianos, os lipopolissacarídeos (LPS), induzem forte resposta imunológica (LI J, BILLIAR TR 1999).

Clinicamente, a sepse causa diversas alterações metabólicas e teciduais (NUNES FB et al 2003; CHEN HW et al 2004), entre elas, uma das mais importantes é a despolarização da membrana da mitocôndria, tornando a fosforilação oxidativa incompleta e conseqüente queda da síntese de ATP. Esses eventos alteram a estrutura da membrana mitocondrial, sua permeabilidade e o fluxo de íons, provocando o *swelling* (FERRI D et al 2005; WILSON JD, BIGELOW CE, CALKINS DJ 2005; JOHNSON LJ et al 2002; BARNARD PJ et al 2004). Alguns poros da membrana mitocondrial estão abertos à difusão de metabólitos, porém não de proteínas, como a citocromo C. A saída de citocromo C e de outras moléculas pró - apoptóticas para o citosol só ocorre com o aumento massivo do *swelling* e a conseqüente ruptura da membrana mitocondrial levando à morte celular por necrose e/ou apoptose (MASUBUCHI Y, SUDA C, HORIE T 2005, KIM JS et al 2004, BRUSTOVETSKY N 2002).

Quando há alteração nas funções das mitocôndrias, ocorre a interrupção da respiração aeróbica, o acúmulo de ácido lático e corpos cetônicos, interferindo na síntese de ATP e aumentando a concentração de espécies reativas de oxigênio (EROs) (CHEN HW et al 2004; VENDITTI P, ROSA RD, MEO SD 2004; KIM JS et al 2004), como por exemplo, o aumento excessivo de óxido nítrico (NO), produzido no estado de sepse.

O NO é sintetizado pelas enzimas óxido nítrico sintetases (NOS), que catalisam a oxidação de um nitrogênio guanidínico da L-arginina para formar óxido nítrico e citrulina. É uma molécula envolvida em várias funções fisiológicas, podendo exercer efeitos deletérios quando produzida em excesso (NISOLI E et al 2004).

Foram identificados quatro tipos de NO sintetases. As constitutivas, presentes em células endoteliais, células do tecido nervoso (eNOS e nNOS, respectivamente) e, recentemente descoberta, a mitocondrial (mtNOS) que é uma isoforma da nNOS, presente em muitos outros tecidos. A indutível (iNOS), presente nas respostas citotóxicas, é a única independente de cálcio, podendo resultar no aumento de distúrbios na sepse (PERSICHINI T et al 2005; ELFERING SL, SARKELA TM, GIULIVI C 2002; CARRERAS MC et al 2004).

Duas EROs, O_2 e NO, são as primeiras a serem produzidas e continuamente formadas na mitocôndria. Outras espécies reativas como H_2O_2, ONOO°, HO°, ROO$^{\circ}$, e $O_2^{-\circ}$, são derivadas da primeira produção de O_2 e NO. O_2 é produto da transferência de elétrons da cadeia respiratória, através da autoxidação de ubisemiquinona. Aqui, o NO é o produto da ação enzimática da mtNOS, que requer NADPH, arginina, O_2 e Ca_2/calmodulina.

Essas espécies reativas de oxigênio reagem com uma variedade de componentes celulares, entre eles a L-tirosina, que pode ser convertida parcialmente a 3-nitro-L-tirosina quando exposta a radicais NO (VAN DER VLIET A et al 1995), estando essa nitrotirosina correlacionada a várias patologias (GOLE MD et al 2000; HENSLEY K et al 1998; ISCHIROPOULOS H et al 1998; SASAKI S et al 2000; SMITH MA et al 1997). Esses caminhos envolvem também a nitração pelo peroxinitrito (BECKMAN JS 1994) ou a ação catalítica de heme-peroxidases usando peróxido de hidrogênio e nitrito (BRENNAN ML et al 2002, EISERICH JP et al 1998).

A interação direta entre NO e citocromo c oxidase, causa inibição da cadeia respiratória. O NO oxida ubiquinol na cadeia respiratória, reage com superóxido para formar peroxinitrito, o qual inibe o complexo I (CARRERAS MC et al 2004), podendo inibir também o complexo II/III, citocromo oxidase (complexo IV) (NISOLI E et al 2004), ATP sintase, creatina quinase e provavelmente outras enzimas (BROWN GC, BORUTAITE V 2002). O NO também estimula a guanilil ciclase que aumenta GMPc e GMPc – dependente, na inibição da permeabilidade de poros mitocondriais (KIRKEBOEN KA, STRAND OA 1999; LI J, BILLIAR TR 1999). Um dos mecanismos da citotoxicidade do NO está no dano causado nos centros de Fe-S (um dos quatro complexos enzimáticos da cadeia respiratória) inibindo irreversivelmente a respiração mitocondrial. O ataque de resíduos de prolina, lisina e arginina pelas espécies reativas de oxigênio na presença de ferro reduzido (Fe^{2+}), resulta na carbonilação de sítios dessas proteínas (DONNEA ID et al 2003; KANTROW et al 1997).

Infravermelho e Mitocôndrias

As mitocôndrias e seus complexos como, proteínas Translocásicas da Membrana Externa (TOM – *Translocase Outer Membrane*), Creatina Quinase Mitocondrial (mtCK), Citocromo c oxidase e transportador de ADP/ATP, têm sido analisados por infravermelho através de vibrações moleculares representadas em sua região média (400 – 4000 cm^{-1}), principalmente nas regiões das bandas de amidas I (1615 –1700 cm^{-1}) e II (1500 -1600 cm^{-1}) referentes aos grupamentos C=O e N-H, respectivamente, constituintes também das proteínas (OBERG KA, FINK AL 1998; VERMETTE P et al 2003; ZANDOMENEGHI G et al 2004).

Estruturas TOM da mitocôndria têm sido determinadas por infravermelho, juntamente com outras tecnicas. As estruturas secundárias de TOM40 (fração do complexo protéico TOM) foram determinadas por FTIR por conter aproximadamente 31% de folha β-pregueada, 22% α-hélices e 47% de outras estruturas. Estruturas de α-hélices estão localizadas em 1650 cm^{-1}, entre 1645 cm^{-1} - 1640 cm^{-1} estão estruturas ao acaso e entre 1630 -1625 cm^{-1} estruturas folha β-pregueada. Em 1695 cm^{-1} temos estruturas caracterizadas por folha β-pregueadas antiparalelas (AHTING U et al 2001).

As isoenzimas Creatinas Quinases (CK) catalisam reversivelmente a transferência dos grupos fosfato da fosfocreatina para o ADP, regenerando ATP. Estudos mostram que alterações na fluidez da membrana mitocondrial podem ser induzidas pela CK mitocondrial (mtCK), influenciando na função da mitocôndria. Resultados de estudos feitos com lipossomos através da espectroscopia de infravermelho, juntamente com outras técnicas, mostraram que a diminuição na fluidez das membranas pode estar relacionada ao acoplamento funcional entre a fosforilação oxidativa, adenina nucleotídeo translocase, porinas e mtCK. O aumento na rigidez de microdomínios fosfolipídicos da membrana mitocondrial pode perturbar as enzimas inseridas nessas membranas, afetando suas funções. Além disso, mudanças na fluidez da membrana da mitocôndria podem contribuir para explicar o envolvimento da mtCK na formação e na função da permeabilidade dos poros dessa organela, envolvidos no processo de apoptose, abrindo novas perspectivas na função fisiológica

desta enzima na célula. Alterações na região de amida I estiveram presentes entre 1630-1660 cm-¹. O aumento do pico na região de 1616 cm-¹ foi concomitante com a diminuição das estruturas α-hélices e β-folhas representadas pela diminuição dos picos em 1651 e 1636 cm-¹ (GRANJON T et al 2001).

Citocromo c oxidase (CcO), enzima responsável pela oxi-redução na cadeia respiratória da mitocôndria, como também em muitas bactérias, catalisa eficientemente a conversão do oxigênio em água. Nesse processo, o movimento de cargas contribui para o gradiente eletroquímico através da membrana. Averiguar a transferência de elétrons, a bomba de prótons e a química do oxigênio, é essencial para entendermos essa máquina molecular. Mudanças vibracionais em resíduos individuais da CcO, em diversas condições, têm sido investigadas por infravermelho. Três regiões foram distinguidas: a de 1690 cm-¹ -1620 cm-¹, a de 1560 cm-¹ -1520 cm-¹ e a região entre 1100 cm-¹ - 1000 cm-¹. As bandas com picos positivos na região de amida I incluem sinais predominantes das estruturas de α- hélices em 1650 cm-¹. Os sinais em 1698 e 1636 indicaram reorganizações de estruturas de β- folhas e os sinais em 1658 cm-¹ representam mudanças em estruturas de α- hélices (RITTER et al 2003; NYQUISTA RM et al 2001; TSUBAKI M, YOSHIKAWA S 1993).

O transportador de ADP/ATP está localizado no interior da membrana mitocondrial, formando ATP a partir do ADP citosólico. Estudos feitos por FTIR, a partir da análise de amidas I e II presentes nesse transportador tem auxiliado para um maior conhecimento destes compostos protéicos e suas funções (FONFRÍA VAL et al 2003). O carboxiatractilosídeo, um inibidor do funcionamento do transportador, de ATP/ADP mitocondrial de *Saccharomyces cerevisiae*, mostra que 60-70 % dos aminoácidos analisados são de α-hélices e estruturas desorientadas, sendo coerente com seis modelos de hélices transmembrânicas. Comparações entre diferentes amostras indicam que tanto os lipídeos podem induzir mudanças em proteínas (diminuição em β-folhas e aumento de estruturas desordenadas), como proteínas podem induzir mudanças em lipídeos (fortes ligações de hidrogênio de grupos C=O dos lipídeos) (LORENZ VA et al 2001).

Ricchelli et al (2001), suspenderam mitocôndrias hepáticas de ratos em diferentes meios, selando os poros das organelas com diferentes compostos, impedindo o trânsito de substâncias do meio através da membrana. A análise feita por infravermelho das amidas I e II da membrana das mitocôndrias, mostrou diferença significativa entre os espectros das seladas em comparação às não seladas nos diferentes meios de suspensão. A análise feita pela espectroscopia de infravermelho da amida I da membrana da mitocôndria em meio contendo sacarose mostrou diferenças relevantes, sendo mais proeminentes os picos localizados em 1658 cm-¹ (estruturas de α-hélices) e 1637 cm-¹ (estruturas β-folha) em comparação aos outros meios. Sendo assim, os pesquisadores concluíram que as interações entre os poros da membrana mitocondrial e os diferentes meios de suspensão podem modificar a estrutura conformacional desses poros (RICCHELLI F et al 2001; RICCHELLI F et al 2003).

Baseado nos dados acima concluiu -se que a espectroscopia de infravermelho possibilita analisar as mais diversas matérias orgânicas e caracterizar individualmente os espectros para cada

uma delas. Sendo assim, as pesquisas realizadas anteriormente somente com substâncias químicas passaram a ser ampliadas aos modelos biológicos, facilitando e aperfeiçoando as técnicas de diagnóstico e prognóstico de doenças.

OBJETIVOS

Geral

Estudar as alterações em mitocôndrias hepáticas causadas pela sepse induzida em ratos (*Rattus novergicus* – var. Wistar) através do FTIR-ATR.

Específicos

- Avaliar modificações moleculares nos espectros de infravermelho das mitocôndrias hepáticas e comparar os resultados entre os ratos do grupo séptico e os demais grupos (controle, controle anestesia e sham);
- Determinar as concentrações de proteínas totais das mitocôndrias hepáticas para averiguar variação significativa ou não em suas concentrações que possam interferir nos espectros;
- Determinar as concentrações séricas de óxido nítrico para averiguar possíveis variações no estresse oxidativo.

ARTIGO CIENTÍFICO

A Ser Submetido a Biochimica et Biophysica Acta

INVESTIGATIONS OF MOUSE HEPATIC MITOCHONDRIA IN SEPSIS USING FTIR-ATR

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Running title: Infrared and Mitochondria in Sepsis

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Summary

Infrared spectroscopies characterize tissues and fluids at a molecular level, and the spectra obtained are characteristic, representing a fingerprint. In recent decades, infrared has been used in several areas of knowledge. Currently, it is employed in the prognosis and diagnosis of diseases, in experimental models and biological systems. Sepsis, a systemic inflammation in reaction to infection, is responsible for a great number of deaths in Intensive Care Units (ICUs). It provokes numerous alterations in the cellular metabolism and the concentration of free radicals surpasses that of anti-free radicals, which may lead to cellular apoptosis or death through necrosis. Nitric Oxide (NO) is a marker of free radical formation and may cause serious damage to the mitochondria, impeding their functioning.
In our study, we analyzed hepatic mitochondria alterations in a model of oxidative stress (sepsis) by FTIR-ATR. Amide I e II bands were analyzed together with the action of free radicals on the organelle. The increase in the amide I peak and increase of levels NO in serum of septic groups in relation to others groups (control, control anaesthesia, sham) was correlated. This significant increase in amide I in septic group represent an increase in C=O groups characteristics proteins α and β unsaturated. Thus being, infrared was capable to differentiate alterations in spectrum of all groups.

INTRODUCTION

The main components of biological membranes named, proteins, lipids, nucleic acids and sugar, show their highest infrared spectral absorption in the bands that refer to the bonds N-H, C=O, C-H and P=O, in the intermediate region, including the interval from 400 – 4000 cm^{-1} [1, 2, 3]. Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR) is a well-established standard method that enables the identification and characterisation of these samples [4, 5].

Sepsis, a systemic inflammation in response to infection, has been the target of many studies, as it is responsible for a great number of deaths in ICUs. This syndrome provokes numerous/uncountable alterations in cellular metabolism mainly in the mitochondria, organelles responsible for most of the cellular energy supply [6, 7, 8]. Thus, alterations occur in the mitochondrial membrane, to its permeability and fluidity, as well as ions transport [9, 10, 11, 12, 13]. This situation favours the generation of reactive species that oxidise amino acid residue, causing carbonylation of these proteins [7, 14].

The purpose of the present study is to analyse alterations in hepatic mitochondria as a result of sepsis in rats [*Rattus novergicus* – var. Wistar), based on molecular modifications in the infrared spectra in the amide I and II regions, corresponding to links C=O and N-H, respectively, present organelle's proteins.

MATERIALS AND METHODS

Experimental Model

Ethical principles regarding manipulation of animals as determined by the Research Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) were respected. Animals were fed with commercial chow (Nuvilab - CR1) for up to twelve hours and water for six hours prior to the experiment, and the light/dark cycle was respected.

Forty-six male rats (*Rattus novergicus* – var. Wistar), from the Biotherium of the Faculty of Biosciences of the PUCRS, aged between 60 and 90 days, weighing approximately 220g (+/- 20g) were used for the experience. Their livers (5-10g) were immediately removed, their blood collected and the serum was separated.

Animals were divided into four groups: Control (n= 16), rats submitted to neither the surgical process nor anaesthesia; Control Anaesthesia (n=12), only received anaesthesia; Sham (n=9), rats submitted to anaesthesia and opening of the peritoneal cavity; and Septic (n=10), submitted to anaesthesia and opening of the peritoneal cavity with the induction of sepsis. The anaesthesia, administered via intraperitoneal (i.p.) at a dose of 2mg/g weight of Ketamine Chlorohydrate 5% / Xylazine Chlorohydrate 2.3g (3:1).

Sepsis was induced by means of *Cecal Ligation and Puncture* (CLP), in which two cuts were made at different points of the colon, permitting faeces to leak out [15, 16]. All animals were sacrificed twelve hours after the initiation of surgical procedures and administration of anaesthesia.

Mitochondria were extracted following procedures previously described [17, 18, 19], with some modifications as follow.

Isolation mitochondria

A differential centrifugation speed was used to isolate mitochondria. After animal scarification, livers (5-10g) were immediately removed and chopped in 20mL of medium containing 250mM sucrose, 1,0mM EGTA and 10mM HEPES-KOH, pH 7.4, homogenized three times for 15 seconds at an interval of 1 minute in a Potter-Elvehjem. The homogenized product was centrifuged at 2500rpm at 4°C for 10 minutes, and the supernatant was re-centrifuged at 10,500rpm for 10 minutes at 4°C. Pellet was suspended in 10mL of medium containing 250mM sucrose, 0,3mM EGTA and 10mM HEPES-KOH, pH 7.4, and later centrifuged at 6000rpm for 15 minutes at 4°C. Pellet was suspended in 10mL of medium containing 250mM of sucrose and 10mM of HEPES-KOH, pH 7.4. Mitochondrial protein content was identified by biuret reaction [20].

Infrared Spectroscopy

For the background plate 1,0mL of medium containing 250mM sucrose and 10mM of HEPES-KOH, pH 7.4, was used to analyse only the mitochondria and be able to discount the medium in which they were found as a blank control

Within four hours of removal, 1,0mL of suspension containing the hepatic mitochondria was placed on a ZnSe optical plate and analysed using infrared spectroscopy. The entire spectrum (4000 – 400 cm-1) was obtained by means of the ATR technique, using a Perkin-Elmer Spectrum One spectrometer (Perkin Elmer, Uberlingen,Germany) with a horizontal ATR device (with angle 45°). Four scans were performed with a resolution of 4cm-¹. Scans were interpolated in the area of interest. In order to better analyse amide I and II peaks, correction programmes supplied with the equipment system were used (Absorbance, Interactive smooth, auto- X, auto- Y). All spectra were analysed in Absorbance and with attenuation of 52. A comparison was made of the maximum peak values of amides I and II (p.m. amide I / p.m. amide II) [21].

Nitric Oxide

Spectrophotometric detection of serum Nitrate: levels of nitrate (NO3) were determined using a copper covered cadmium reagent (cadmium/copper). The reagent was prepared by previously washing 2-3g of 100 mesh granulated cadmium with 50mL of water milliQ (nanopure) in a 250mL Erlenmeyer, supernatant was discarded, and cadmium washed twice with 50mL of HCl 0,5N. Soon, cadmium was washed with 50mL of water and after with 50mL of 5% copper acetate, in order to cover the cadmium with copper. Cadmium was washed with copper acetate two or three times, the blue solution remaining in contact with cadmium for 60 seconds, saturating cadmium with copper. During this period, cadmium must not turn reddish or black, if this occurs it should be discarded. The excess of copper was quickly removed by four washes with 25mL water. Cadmium covered with copper was washed twice with 30mL of HCl 0,1N and stored in this solution at temperature between 2-8°C. After this process the product is stable for six months [22].

In order to determine total nitrate and nitrite, Griess acid reaction was used for the development of colour after reduction of nitrate by cadmium/copper reagent and proteinisation. In this process, 100µl of serum was added to a microtube containing approximately 0.1mL of cadmium/copper (pre-washed in the same carbonate/bicarbonate buffer and dried with filter paper) and 0.4mL of carbonate/bicarbonate buffer (pH 9,0). After, it was vortexed and incubated at room temperature for 1 hour. Reaction was interrupted with addition of 0.1mL of sodium hydroxide 0.35M, and standing for 10 minutes. It was then centrifuged at 4000g for 10 minutes. 500µl of cleaned supernatant was transferred to another tube, were added 250ul of sulphanilamide 1% (prepared in HCl 3N) and 250ul of N-naftylethylenodiamine (prepared in water) and mixed. After 10 minutes, reaction tubes were read at 540nm against a background containing only reagents [23].

Statistical Analysis

Turkey and Bonferroni statistical tests were used for statistical analysis, by means of One - Way ANOVA with the help of SPSS software version 11.5, with a $p < 0.05$ is considered significant and $p < 0.001$ is considered a lot significant.

RESULTS

Spectra of hepatic mitochondria show the amide I peaks, regarding bonds C=O (1615 –1700 cm^{-1}), and amide II, regarding the bonds N-H (1500 -1600 cm^{-1}). An increase in the amide I peak was observed in the septic group (red line) in comparison with other groups: control, anaesthetised and sham (black lines) in Figure 1.

The visible difference represented by infrared spectrum in the amide I region, of the septic group (red line), is confirmed though the comparison between the average amide I peaks / average amide II peaks, as shown in Figure 2. Average for the control group was 0.905 ± 0.037, for the control anaesthesia group was 0.914 ± 0.051, for the sham group, 0.884 ± 0.044 and for the septic group, 1.007 ± 0.089. This comparison of averages showed a significant difference between septic group $p < 0.001$(**) and the others: control, control anaesthesia and sham; but none between control, control anaesthesia and sham.

Figure 3 shows normalised spectra (black curves) and their inverted derived seconds (red curve) obtained from mitochondria from the control (A), control anaesthesia (B), sham (C) and septic groups (D), respectively. In the deconvoluted spectra, at the peak of amide I, some “minor peaks” arose while others disappeared. In the control group, four peaks were formed, located at: 1660 cm^{-1}, 1650 cm^{-1}, 1639 cm^{-1}, 1625 cm^{-1}. In the anaesthetic and sham groups, two peaks disappeared (1660 cm^{-1} and 1639 cm^{-1}), with one remaining at 1650 cm^{-1} and another at 1625 cm^{-1}. In the spectrum of the septic group, the peak located at 1660 cm^{-1} disappeared, followed by the presence of those at 1650 cm^{-1}, 1639 cm^{-1} and1625 cm^{-1}.

As Figure 4 show, there was no significant difference in the total protein concentration of the hepatic mitochondria between groups. Values were 3.969 ± 0.217 for control group, 3.433 ± 0.104 for control anaesthesia group, 3.389 ± 0.265 for sham group and 3.69 ± 0.164 for septic group.

Serum levels of NO, a free radical formation marker, were 15.294 ± 3.262 for control group, 14.367± 4.238 for control anaesthesia group, 17.011 ± 4.853 for sham group and 24.19 ± 6.479 for septic group, demonstrating a significant difference with $p < 0.05$ (*) between septic and sham groups, and of $p < 0{,}001$ (**) between septic group and control and control anaesthesia groups (Figure 5). These results show evidences of an increase in the induction of NO in animals 12 hours after sepsis,

as the levels of NO were found to be significantly elevated in this group when compared to the control, control anaesthetized and sham groups.

DISCUSSION

In our study, we used Fourier Transform Infrared Spectroscopy in a model of oxidative stress (sepsis) in hepatic mitochondria in order to analyse the amide I and II bands, the action of free radicals on the membrane of this organelle and its correlation with the increase in the amide I peak and the serum levels of nitric oxide. Amide bands I and II refer to the molecular vibrations of the groups C=O and N-H, respectively [24, 25].

Sepsis is brought about by a complex series of cellular and biochemical events, including liberation of inflammatory cytokines, increasing oxidative stress and induction of nitric oxide synthesis (iNOS) [26]. We found evidence of an increase in the induction of NO in animals 12 hours after sepsis (Figure 5). This is in line with previously reported studies using the same experimental animal model, though at 16 hours following the induction of sepsis [27]. Nitric Oxide Synthase (NOS) is present in many cells, though the iNOS isoform is the only one that is independent of calcium and dependent on TNF-α and can result in cytotoxicity in sepsis. The objective in evaluate NO into the serum was to estimate whether the different groups were at different stages of oxidative stress.

NO is a free radical formation marker, enzymatically formed, or not, in the tissues [8]. This molecule is involved in several physiological functions, and may cause damage when in excess [28]. It is formed by the NOS, which catalyzes the oxidation of a guanidine hydrogen of L-arginine to from nitric oxide and citruline.

Reactive species of oxygen react with a variety of cellular components, among them L-tyrosine that can be partially converted to 3-nitro-L-tyrosine when exposed to NO radicals [29], and this nitrotyrosine is correlated in a number of pathologies [30, 31, 32, 33, 34]. These paths also involve nitration also by peroxynitrite [35] or the catalytic action of heme peroxidase using hydrogen peroxide and nitrite [36, 37].

One of the mechanisms of cytotoxicity of NO is the damage caused in the Fe-S centers, which irreversibly inhibits mitochondrial respiration, preventing the organelle from correctly generate the energy necessary for the cell, and so favouring anaerobiosis. This favours an increase in cell membrane permeability. The attack of the reactive oxygen species on the proline residues, lysine and arginine in the presence of reduced iron (Fe2+), results in carbonylation of these proteins [27, 38].

In the last decade, many papers were published that reported increased levels of these carbonylated proteins in numerous pathologies and the evolution of diseases. Proteins containing CO groups are elevated in situations of oxidative stress, as occurs in sepsis,

neurological diseases, chronic juvenile arthritis, in patients with chronic kidney failure or haemodialysis, and in so many other pathologies [39, 40].

In previous studies, in the same sepsis induction model (CLP), increases in the concentration of carbonylated proteins from hepatic mitochondria were found [27]. We monitored, at an interval of 1000 - 1400 cm^{-1} of the infrared, the most relevant differences found in these samples, which were found in the amide I region (1700–1600 cm^{-1}) referring to the carbonylic groups (C=O) also present in the proteins of mitochondria.

In our experiment, we analyzed the levels of total mitochondrial protein (Figure 4) by means of the Biureto method [20, 41], and no significant difference in its concentration was found between different groups ($p > 0,05$). Being thus, the increase in C=O groups in the septic group is thought to be the result of the formation of these groups and no distinctions in the concentration of total proteins in the septic group.

Analysis of these proteins with CO groups as biomarkers of oxidative stress offers advantages, in comparison to certain oxidative products, since CO groups are formed early and are chemically stable, and as such are useful for detection and storage. Moreover, these carbonylated proteins take hours or days to be degraded and eliminated by the organism, while lipid peroxidation products take only a few minutes. The stability of the stored carbonylated proteins stored at - 80 °C is approximately 3 months [39, 40].

Clinically, sepsis causes several metabolic and tissue alterations [6, 7], altering the structure of the mitochondrial membrane and, consequently, the permeability, the transport of ions and organelle function. This process of alterations in mitochondrial permeability and its consequences is known as *swelling* [11, 12, 13].

Ricchelli et al, in a model of swelling, suspended hepatic mitochondria from rats in different media sealing the pores of the organelles with different compounds and analyzing infrared amide I and II bands. The second derivatives from their spectra showed that there was a significant difference between the sealed organelles and non-sealed in the different suspension media [24, 25].

In Figure 3 the normal and deconvoluted spectra, peaks were perceived in the control (A), control anaesthesia (B), sham (C) and septic groups (D). Thus, evaluating the spectra, we were able to distinguish spectral patterns for the different groups of mitochondria, which allowed us to outline a profile of mitochondrial normality and evaluate which are altered and correlate them with the severeness of the disease, or, further, identify which alteration the anaesthesia could provoke.

In conclusion, the infrared showed, by the increase in the amide I peak, the significant increase in the formation of the C=O groups of carbonylated proteins, in the membrane of the hepatic mitochondria from the septic group in relation to the other groups. This was evaluated

by means of the comparison between the averages of the maximum peaks in the amide I over the amide II bands. Furthermore, the dosage of NO makes it possible to evaluate significant differences in its concentrations in the sepsis.

The fact that no studies were found that correlated alterations in the hepatic mitochondria in this model of sepsis with the aid of FTIR, gives the present study an innovatory character in the analysis of these organelles in experimental models of oxidative stress.

Acknowledgements

We are grateful to the Chemistry of Natural Products Laboratory of the Faculty of Chemistry and the Biophysics Laboratory of the Faculty of Biosciences of PUCRS.

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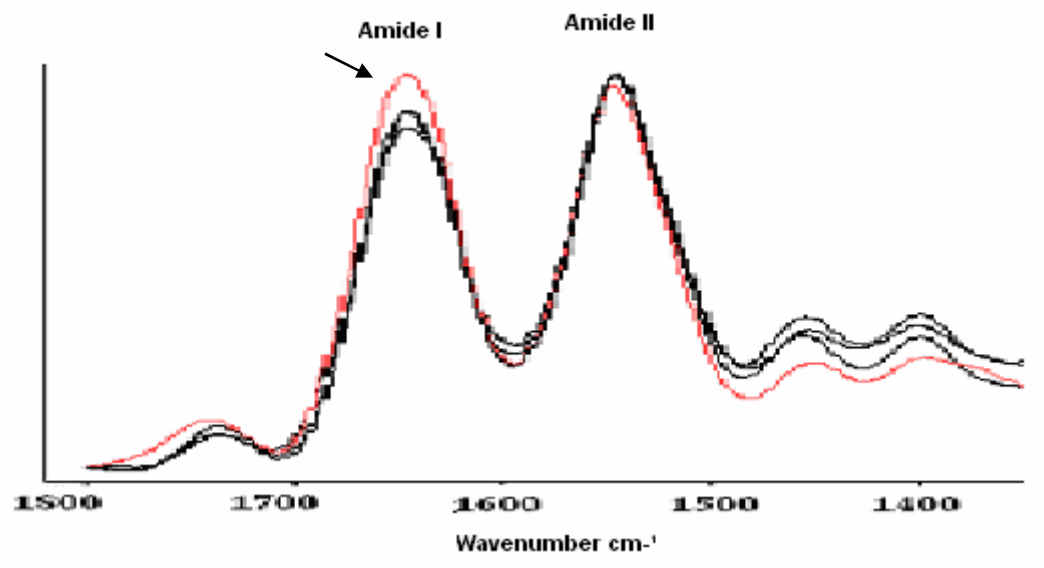
Amide I
Amide II
1800
1700
1600
1500
1400
Wavenumber cm-1

Figure 1: Infrared Spectroscopy of mitochondria, showing amides I and II peaks and the increase in the amide I peak in septic group (in red).

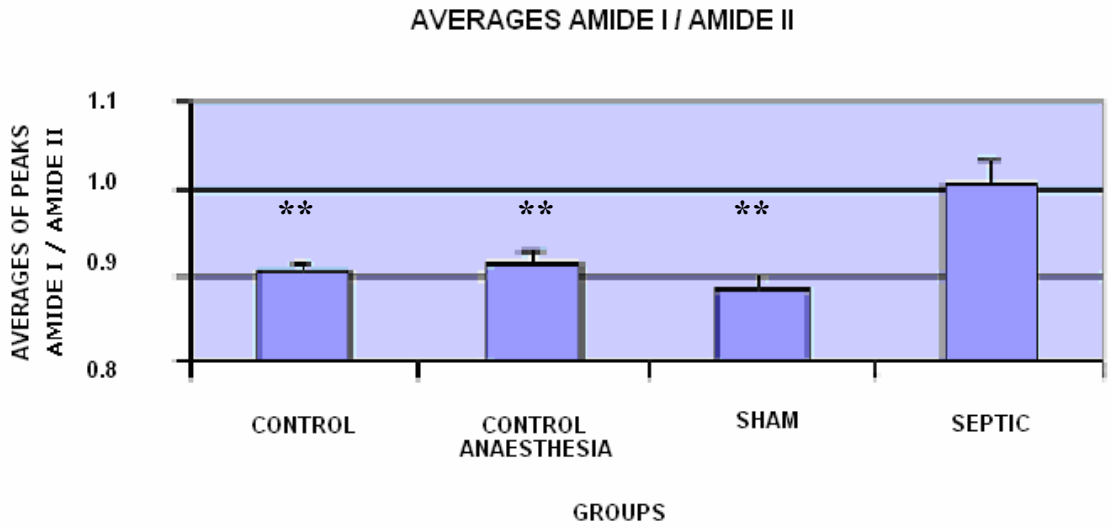
AVERAGES AMIDE I / AMIDE II
AVERAGES OF PEAKS
AMIDE I / AMIDE II
1.1
1.0
0.9
0.8
**
**
**
CONTROL
CONTROL
ANAESTHESIA
SHAM
SEPTIC
GROUPS

Figure 2: Comparison between averages of amide I /amide II peaks, showing a significant difference of septic group compared to other groups: control, control anaesthesia and sham ($p < 0.001$). Difference when comparing groups control, control anaesthesia and sham was not significant ($p > 0.05$).

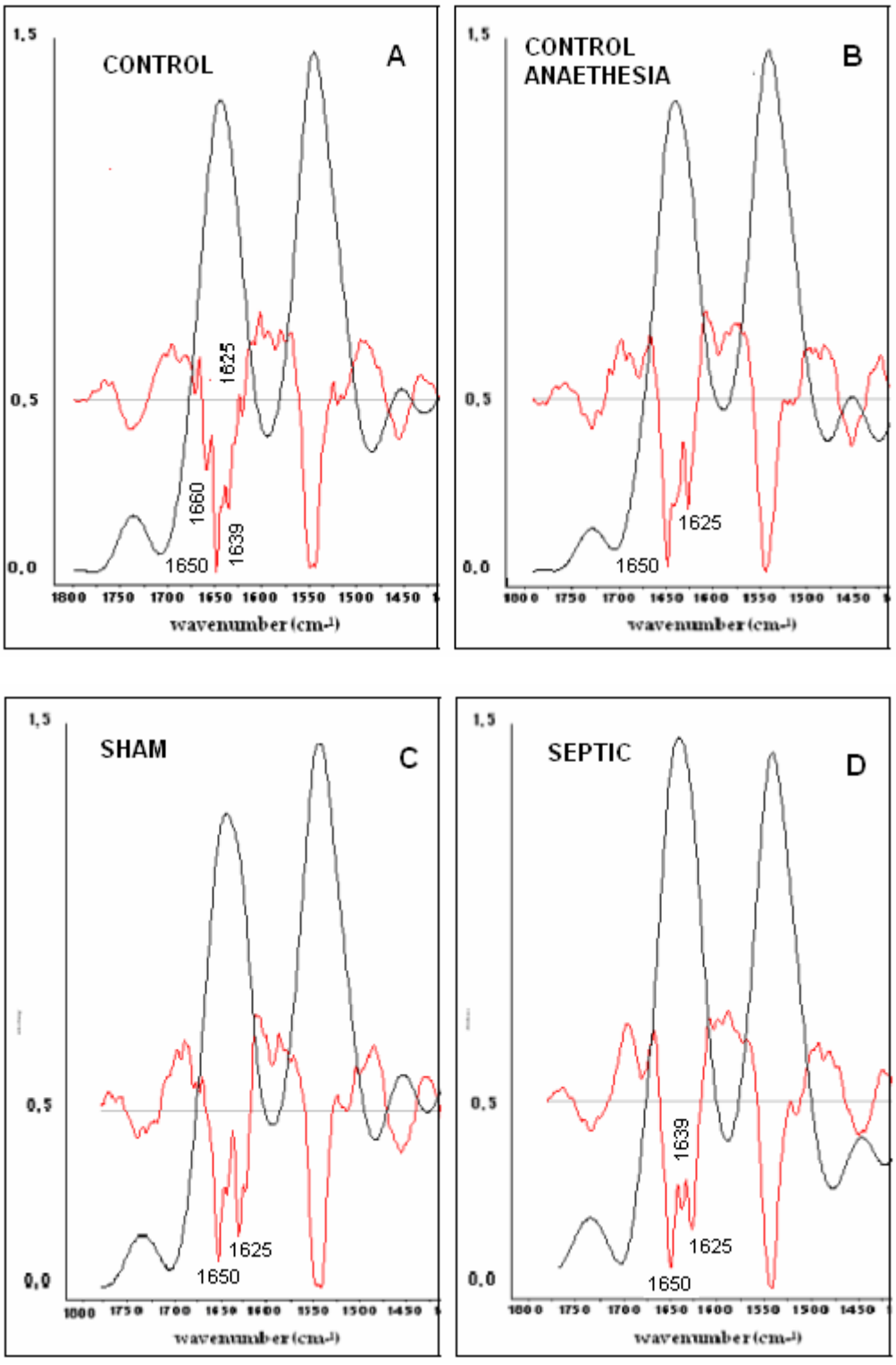
CONTROL
A
1,5
0,5
0,0
1625
1660
1639
1650
1800 1750 1700 1650 1600 1550 1500 1450
wavenumber (cm-1)
CONTROL
ANAETHESIA
B
1,5
0,5
0,0
1625
1650
1800 1750 1700 1650 1600 1550 1500 1450
wavenumber (cm-1)
SHAM
C
1,5
0,5
0,0
1625
1650
1800 1750 1700 1650 1600 1550 1500 1450
wavenumber (cm-1)
SEPTIC
D
1,5
0,5
0,0
1639
1625
1650
1800 1750 1700 1650 1600 1550 1500 1450
wavenumber (cm-1)

Figure 3: Infrared Spectrum (black curves) and their inverted derived seconds (red curve) in absorbance obtained from mitochondria of control (A), control anaesthesia (B), sham (C) and septic groups (D).

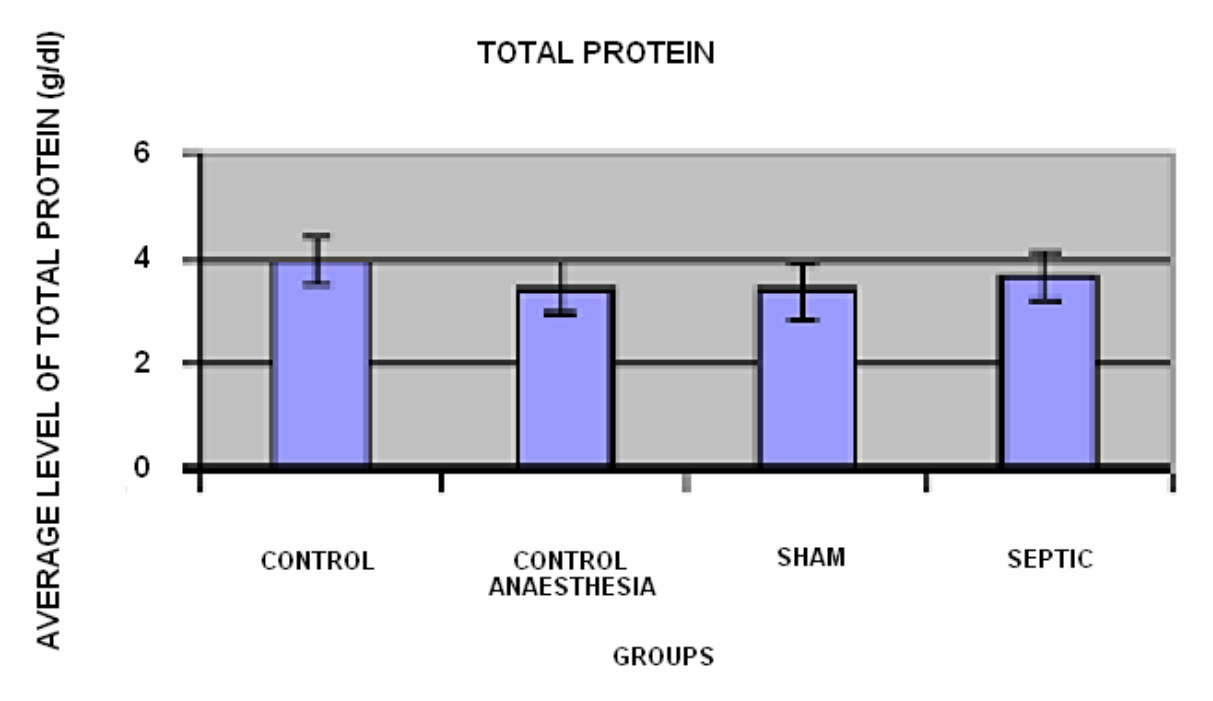
TOTAL PROTEIN
AVERAGE LEVEL OF TOTAL PROTEIN (g/dl)
6
4
2
0
CONTROL
CONTROL ANAESTHESIA
SHAM
SEPTIC
GROUPS

Figure 4: Average concentration of total protein, showing there was no significant difference among groups.

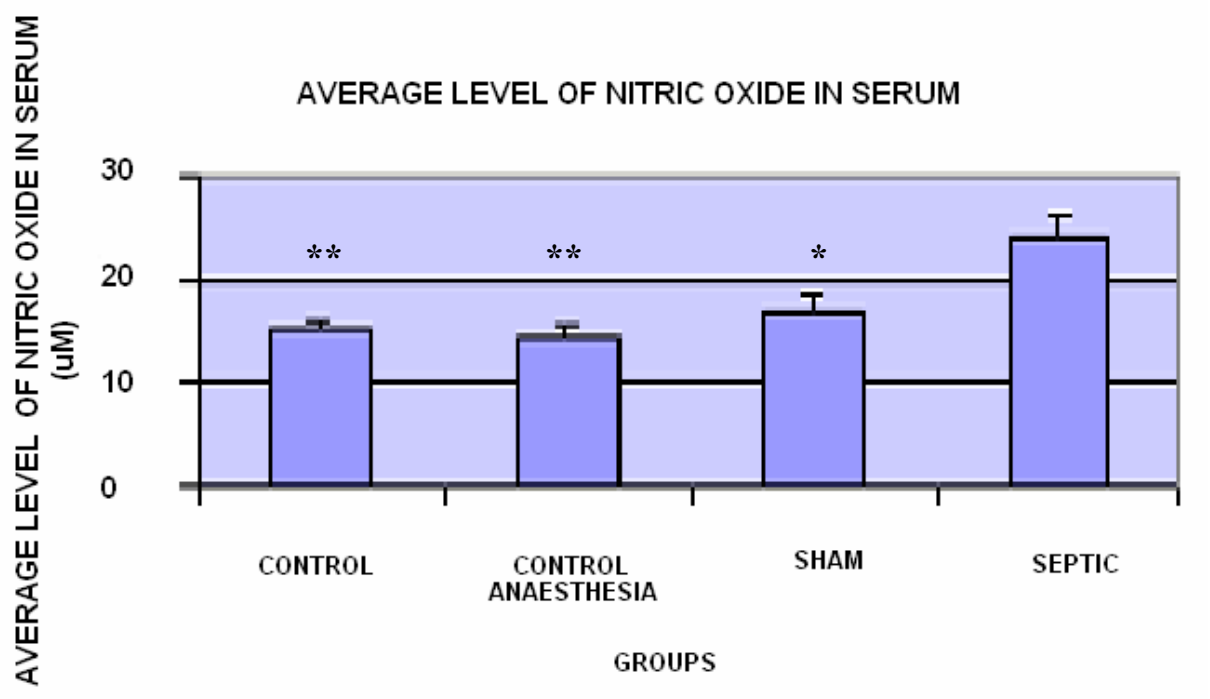
AVERAGE LEVEL OF NITRIC OXIDE IN SERUM
AVERAGE LEVEL OF NITRIC OXIDE IN SERUM (uM)
30
20
10
0
**
**
*
CONTROL
CONTROL ANAESTHESIA
SHAM
SEPTIC
GROUPS

Figure 5: Concentration of NO in different groups: control, control anaesthesia, sham and septic, showing a significant difference when comparing septic and other groups.

CONCLUSÃO

Em nosso trabalho pôde-se perceber o aumento significativo na banda de amida I relacionado ao aumento na formação de grupos C=O no grupo séptico comparando este com os demais grupos (anestesia, sham e controle). Sendo assim, a sepse alterou a relação entre as bandas de amida I / amida II. Além disso, a técnica de IV foi capaz de diferenciar alterações espectrais nos diferentes grupos.

O aumento significativo de NO sérico no grupo séptico em comparação aos demais grupos nos permitiu verificar diferença significativa no estresse oxidativo entre os grupos, sendo possível correlacionar estes níveis de NO com o aumento da banda de amida I das mitocôndrias hepáticas característicos de grupamentos C=O de proteínas α e β insaturadas.

PERSPECTIVAS

Apesar de não ter sido encontrado na literatura o uso da Microscopia Eletrônica de Varredura (MEV) em mitocôndrias, esta foi feita para posterior comparação com a Microscopia Eletrônica de Transmissão (MET), para que sejam analisadas as alterações possíveis na membrana externa desta organela, em seu diâmetro e em suas estruturas internas.

Além de nossas pretensões em fazer a MET destas organelas, também almejamos dosar aldeídos nas mitocôndrias hepáticas por HPLC, pois esses são produtos da lipoperoxidação da membrana dessa organela devido ao ataque de radicais livres na sepse.

Pretende-se também, criar um índice através da relação amida I / amida II, para avaliar a gravidade da sepse.

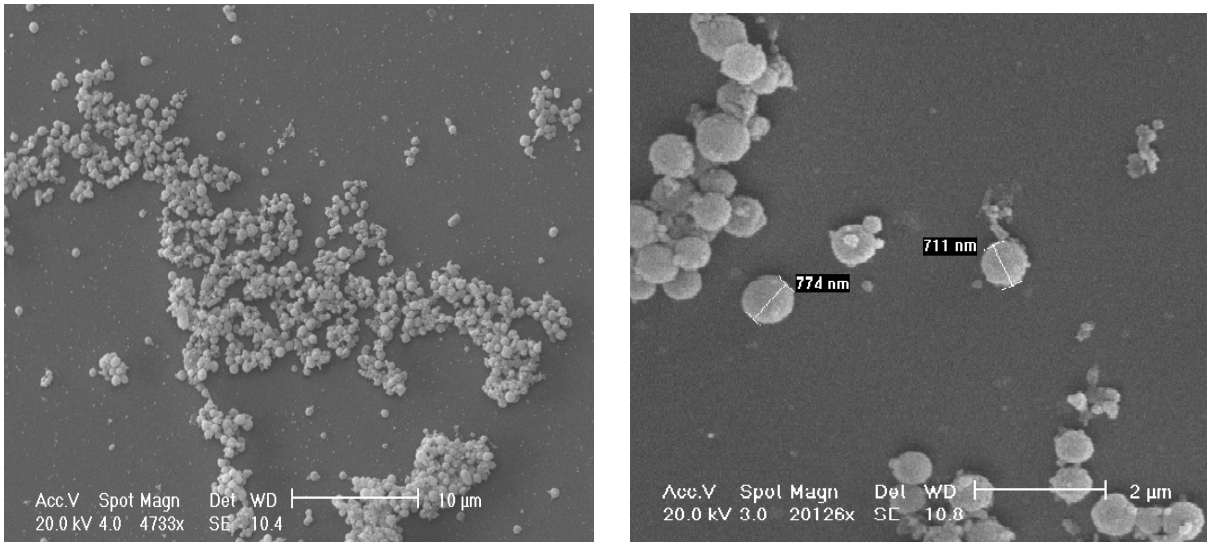

Foto por MEV de mitocôndrias hepáticas

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