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ESTRESSE NITRO-OXIDATIVO INDUZIDO POR SORO URÊMICO *IN VIVO* E *IN VITRO*

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ESTRESSE NITRO-OXIDATIVO INDUZIDO POR SORO URÊMICO *IN VIVO* E *IN VITRO*

Dissertação apresentada ao Curso de Pós-Graduação em Ciências da Saúde, da Pontifícia Universidade Católica do Paraná, como requisito parcial para a qualificação ao título de Mestre.

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Existem pessoas em nossas vidas que nos deixam felizes pelo simples fato de terem cruzado o nosso caminho. Algumas percorrem ao nosso lado, vendo muitas luas passarem, mas outras apenas vemos entre um passo e outro. A todas elas chamamos de amigo.

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RESUMO

Pacientes de doença renal crônica (DRC) apresentam maiores riscos de desenvolver doença cardiovascular (DCV) que pacientes com função renal normal. Dentre vários fatores que contribuem para patologia vascular, inflamação, calcificação e estresse oxidativo têm sido considerados fatores de risco não tradicionais. A uremia, peculiar somente à população DRC, pode representar um novo fator. Neste trabalho, demonstramos uma calcificação vascular acentuada em pacientes DRC jovens, assintomáticos e com poucos fatores de risco tradicionais de DCV. A calcificação foi observada em 68% dos pacientes DRC, comparada com 7% do grupo controle (chi2 =12,4; p<0,0005). E a intensidade da marcação de nitrotirosina mostrou-se elevada no grupo DRC (62,18±4,26), quando comparado ao grupo controle (20,21±3,62; p<0,0001), sugerindo que tanto calcificação vascular como nitração protéica podem ser relevantes para a aceleração do processo aterosclerótico, bastante freqüente na DRC. Além disso, como a DRC é uma doença multifatorial, analisamos os efeitos da uremia *per se* em células endoteliais (EC) em cultura. Nossos estudos mostraram que após 3 horas de exposição ao soro urêmico ocorre um aumento da atividade NADPH oxidase (1,55 vez ± 0,23 sobre o tratamento com soro não urêmico; p<0,05), juntamente com uma tendência de aumento dos níveis de GSH intracelular (1,33 ± 0,24 *vs* 0,97 ± 0,19 µmol GSH/µg proteína com soro não-urêmico, p=2,77). Após 6 horas de exposição foi observada uma diminuição da concentração de GSH intracelular (0,77 ± 0,11 µmol *vs*. 1,19 ± 0,02 µmol GSH/µg proteína soro não-urêmico, p=0,02), indicando a instalação de estresse oxidativo. A uremia induziu também uma perturbação na biodisponibilidade do óxido nítrico (NO), diminuindo após 24h (3,84± 0,59 *vs* 8,53 ± 0,47; p<0,05). Paralelamente, foi verificado um aumento de proteínas nitradas (1,34 ± 0,01 *vs.* 1,02 ± 0,01 sobre células sem tratamento, p<0,01) e da expressão das proteínas GRP94 (razão GRP94/_-actina 0,85 ± 0,02 *vs*. 0,57 ± 0,02, p<0,05) e GRP78 (razão GRP78/_-actina 1,15 ± 0,02 *vs*. 0,88 ± 0,08, p<0,05) em 24 h de tratamento, indicando a indução de estresses nitroxidativo e do retículo endoplasmático (RE). Verificamos também que, neste modelo, N-acetilcisteína (NAC) mostrou-se um antioxidante eficaz, inibindo cerca de 60% da atividade NADPH oxidase da EC (0,67 ± 0,15 vez *vs.* 1,55 vez ± 0,23 sobre o tratamento com soro não urêmico; p<0,05) e também diminuindo o estresse nitroxidativo (1,006 ± 0,095 sobre células sem tratamento *vs.* 1,338 ± 0,014 sobre células sem tratamento; p<0,05) e o estresse do RE (razão GRP94/_-actina 0,85 ± 0,02 *vs*. 0,40 ± 0,02, p<0,002 e razão GRP78/_-actina 1,15 ± 0,02 *vs*. 0,68 ± 0,08, p<0,05), dando suporte aos achados clínicos. Em conjunto, os dados aqui apresentados mostram uma correlação positiva entre calcificação e nitração vascular na DRC, indicam que a uremia *per se* é capaz de alterar a homeostasia redox em EC e sugerem os possíveis mecanismos pelo qual NAC tem se mostrado um antioxidante interessante em DRC.

Palavras-chave: Uremia, Células Endoteliais, N-acetilcisteína, Estresses Nitroxidativo, Calcificação.

LISTA DE ABREVIATURAS

AGE: produtos de glicação avançada, do inglês *advanced glication products*

ApoE: apolipoproteina E

ATF6: do inglês *activating transcripti factor 6*

BiP: do inglês *Binding protein*

Ca: cálcio

CEs / ECs: células endoteliais

c-NOS: óxido nítrico sintase constitutiva

DAC: doença aterosclerótica arterial coronariana

DAF: diacetato de diaminoflurosceina

DCFH-DA: 2',7'-diclorodihidrofluoresceína

DCV / CVD: doença cardiovascular

DHE: diidroetidio

DRC / CKD: doença renal crônica

e-NOS: óxido nítrico sintase endotelial

ESRD: do inglês *End stage renal disease*

GRP: Glicoproteína

GSH: glutationa

GSSG: glutationa dissulfeto

H_2O_2: peróxido de hidrogênio

HD: hemodiálise

HE: coloração de hematoxilina-eosina

i-NOS: óxido nítrico sintase induzível

IRE1: Quinase e endoribonuclease serina-treonina

KDEL: seqüência de peptídeos presente em proteínas do reticulo endoplasmático

LVH: hipertrofia ventricular esquerda

NAC: N-acetilcisteína

NADPH: nicotinamida adenina dinucleótido fosfato

NO: óxido nítrico

NOS: óxido nítrico sintase

P: fósforo

PCR / CRP: Proteína C reativa

PERK: do inglês *PKR-like ER kinase*

PTH: hormônio paratireoideano

RAEC: células endoteliais de aorta de coelho

RE: retículo endoplasmático

ROS: espécies reativas de oxigênio, do inglês, *reactive oxygen species*

SFB: Soro fetal bovino

SOD: superóxido dismutase

TFG: Taxa de filtração glomerular

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1. INTRODUÇÃO

1.1. Doença Renal Crônica

A doença renal consiste em lesão renal e perda progressiva e irreversível da função dos rins. Em sua fase mais avançada, chamada Doença Renal Crônica (DRC), os rins não conseguem mais manter a normalidade do meio interno do paciente [1]. O estadiamento da DRC, em estágios de 1 a 5, pode ser determinado de acordo com a taxa de filtração glomerular (TFG), conforme tabela 1, de acordo com a severidade, diagnóstico, tratamento e prognóstico.

Tabela 1: Estágios da Insuficiência Renal Crônica, classificados de acordo com a taxa de filtração glomerular (TFG):

Estágio	*TFG (ml/mim/1,73m^2)*	*Grau de Insuficiência Renal*
0	>90	Inclui pessoas integrantes dos chamados grupos de risco para o desenvolvimento de DRC (diabetes, hipertensão, idosos, familiares de portadores de DRC) que ainda não desenvolveram lesão renal.
1	>90	Corresponde às fases iniciais de lesão renal (microalbuminúria, proteinúria).
2	60 – 89	Corresponde ao início da insuficiência renal; nesta fase o indivíduo não apresenta sinais ou sintomas de doença renal.
3	30 – 59	Os sintomas renais podem se fazer presentes de forma branda, geralmente o indivíduo apresenta somente queixas relacionadas a sua doença de base como diabetes, hipertensão.
4	15 – 29	Presença de sinais e sintomas de uremia (náuseas, vômitos, perda do apetite, emagrecimento, falta de ar, edema, palidez, etc).
5	<15 (ou diálise)	Nesta fase, os sintomas se intensificam e as opções terapêuticas são os métodos de depuração artificial do sangue (hemodiálise ou diálise peritoneal) ou o transplante renal.

Fonte: Adaptado de Romão-Junior 2004

A hipertensão arterial, tabagismo, hipercolesterolemia e obesidade são fatores globais de risco para a saúde e estão fortemente associados à doença renal crônica. Esses fatores, aliados ao crescimento da incidência de diabetes e ao envelhecimento da população, estão determinando um aumento expressivo na freqüência de doenças renais em todo o mundo [2].

Estima-se que existam mais de dois milhões de brasileiros portadores de algum grau de disfunção renal. Atualmente, no Brasil mais de 70.000 pacientes, cerca de 340

pacientes por milhão de habitantes, são dependentes de terapia de reposição renal, seja diálise ou transplante renal, com gasto anual de cerca de dois bilhões de reais [2, 3]. Aproximadamente 18000 novos pacientes com DRC iniciam a diálise, na maioria das vezes hemodiálise, todo ano no Brasil [3]. A síndrome urêmica é a conseqüência da falência renal, e é atribuída à progressiva retenção de um grande número de compostos, chamados de toxinas urêmicas, que em condições normais são excretados pelos rins saudáveis [4].

A sobrevida em longo prazo e a qualidade de vida de pacientes com doença renal crônica é determinada pelas complicações que se desenvolvem durante o curso da doença. A doença cardiovascular (DCV) é a primeira causa de morbidade e mortalidade prematura em doença renal crônica. Já é estabelecido que pacientes com DRC estágio 5 estão no mais alto fator de risco para doença cardiovascular [5], embora pacientes com DRC em estágios de 1 a 4 também apresentem taxas de eventos cardiovasculares mais elevadas que a população em geral .

1.2. Doença Renal Crônica e risco Cardiovascular

Quando comparados à população normal, os pacientes de DRC apresentam maior prevalência de fatores de risco tradicionais para DCV [6]. Mas fatores de risco tradicionais como hiperlipidemia, hipertensão, ou tabagismo não são suficientes para explicar o grande risco cardiovascular destes pacientes. Outros fatores de risco associados a DCV e relacionados à doença renal crônica têm sido considerados fatores de risco "não tradicionais". Estes incluem anormalidades hemodinâmicas e metabólicas decorrentes da disfunção renal. A prevalência de muitos dos fatores não tradicionais aumenta à medida que a função renal diminui [7, 8]. Entre tais fatores destacam-se as dislipidemias, inflamação, infecção, estresse oxidativo, calcificação e a presença de toxinas urêmicas sistêmicas [7-9].

Apesar de todos os fatores não-tradicionais, o risco cardiovascular é ainda muito mais alto nos portadores de DRC, pois provavelmente o acúmulo de toxinas urêmicas, que é o grande diferencial comparado à população normal, possa contribuir para a ocorrência e extensão dos processos de inflamação, estresse oxidativo e calcificação. Estudos mostraram que a nefrectomia em camundongos *knockout* para ApoE acelera a aterogênese pelo acúmulo de toxinas urêmicas [10, 11]. Alterações funcionais e morfológicas do endotélio, estimuladas por vários fatores lesivos ao vaso, têm papel fundamental no processo aterogênico[12]. O aumento de níveis de citocinas, produtos

bacterianos, produtos de glicosilação avançada [13, 14], hiperomocisteinemia [15, 16], hipercolesterolemia [17], lipoproteínas oxidadas [18] e hiperuricemia [19] são alguns dos fatores característicos na DRC que levam a disfunção endotelial. Além disso, o acúmulo de toxinas urêmicas resulta em mudanças qualitativas e quantitativas no endotélio. Dados mostram que a uremia causa disfunção em células endoteliais *in vitro*, especificamente pela alteração da morfologia, da expressão de moléculas de adesão e da taxa de proliferação [17, 20, 21], de acordo com a associação descrita entre desenvolvimento de aterosclerose e proliferação anormal de células endoteliais e musculares lisas [22].

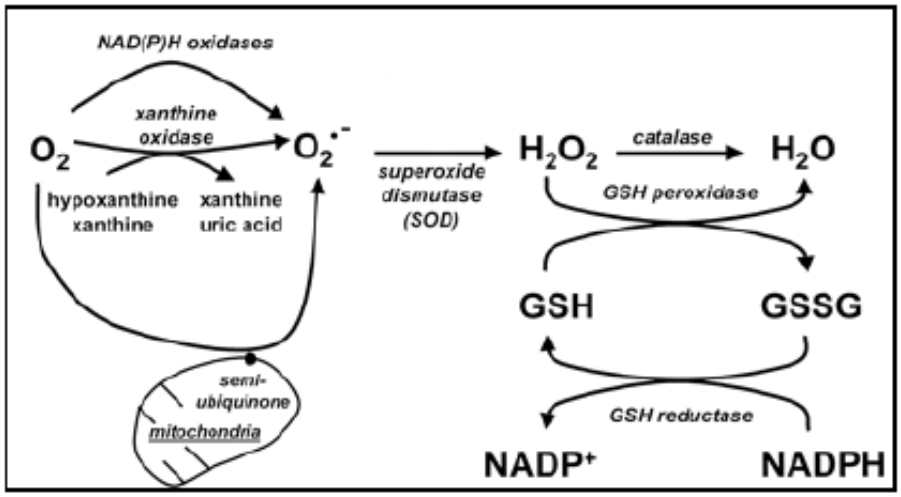
1.2.1. Estresse oxidativo

Estresse oxidativo tem sido definido na DRC como desbalanço entre sistemas pró-oxidantes e antioxidantes, resultando em danos a moléculas e tecidos [23, 24]. Os organismos aeróbios em seu estado natural possuem antioxidantes em quantidades suficientes para reagirem com as espécies reativas de oxigênio (ROS), que são continuamente produzidas pelo metabolismo celular. Quando a produção de ROS excede a capacidade natural dos antioxidantes, ou quando há diminuição dos níveis de antioxidantes no organismo ocorre o estresse oxidativo [25, 26], que compromete a integridade e funcionalidade de proteínas, lipídios, carboidratos e outras biomoléculas. O sistema antioxidante é composto de enzimas, como a superóxido dismutase, glutationa peroxidase, glutarredoxina, tiorredoxina e catalase, como também antioxidantes não enzimáticos incluindo vitaminas (C e E), β-caroteno e a glutationa (GSH) [27, 28].

A glutationa (GSH) é um antioxidante não-enzimático que tem papel catabolizador de peróxido de hidrogenio (H_2O_2) e outros peróxidos através de uma reação enzimática. Na reação da glutationa peroxidase, glutationa (GSH) é oxidada em glutationa dissulfeto (GSSG), que pode ser convertida de volta em GSH pela enzima glutationa redutase em um processo que consome NADPH, conforme figura 1 [23]. A razão entre GSH/GSSG é um indicador do potencial redox celular, e sua diminuição pode ser decorrente do estresse oxidativo em nível celular [16, 24]. Pacientes com doença renal apresentam estresse oxidativo aumentado e podem ter uma diminuição dos níveis de antioxidantes [29]. Diversos estudos já mostraram o estresse oxidativo em eritrócitos e leucócitos de pacientes com DRC, através da razão GHS/GSSG e da expressão e/ou atividade de antioxidantes [30-32]. O estresse oxidativo já foi também

detectado em plasmas de pacientes com DRC pelo conteúdo de tióis, formação de carbonilas e peroxidação lipídica. Tióis têm funções críticas no tampão redox intra e extracelular, via equilíbrio da concentração tiól (S-H) e dissulfeto (S-S) [33]. Vários estudos dosaram a peroxidação lipídica em plasma de pacientes portadores de DRC em diálise, muitas vezes controversos [18, 33-35]. Estudos mostram que a oxidação de tióis e a formação de carbonilas em plasma de pacientes em diálise são significativamente maiores que nos controles [33, 36]. Sendo assim, todos estes estudos mostram que pacientes em hemodiálise apresentam particularmente um elevado estresse oxidativo sistêmico caracterizado em plasma.

Figura 1: Vias da produção de ROS e seu *cleareance* pelo sistema GSH



Fonte: Droge, W, 2002 [23]

Na fisiopatologia vascular, a participação de espécies reativas de oxigênio (ROS) tem sido cada vez mais documentada, produzidas principalmente pela NADPH oxidase e óxido nítrico sintases (NOS) [37-41], flavoenzimas que quando ativadas, catalisam um sistema transportador de elétron que doa elétron para uma molécula de oxigênio, resultando na formação de um ânion superóxido [42]. O complexo NADPH oxidase foi identificado originalmente em fagócitos, onde desempenha papel essencial na defesa contra microrganismos. Esse complexo fagocítico é responsável pelo *burst* oxidativo para gerar grandes quantidades de superóxido. Em células endoteliais a produção de ROS pelo complexo NADPH oxidase é contínua e em baixas quantidades, mesmo em células não estimuladas [43]. Sua ativação é regulada por uma diversidade de estímulos, incluindo agonistas de receptores acoplados à proteína G, como a

angiotensina II, fatores de crescimento, fatores metabólicos como alta glicose, insulina, produtos de glicação avançada (AGEs), LDL oxidado, eventos mecânicos, entre outros [37].

Modelos experimentais com camundongos *knockout* para apolipoproteina E (ApoE) com falência renal forneceram evidências de que o estresse oxidativo desempenha papel importante no desenvolvimento da doença cardiovascular em pacientes com DRC [11]. Satoh e colaboradores mostraram que o complexo NADPH oxidase e a NOS desacoplada são as maiores fontes de estresse oxidativo em ratos com nefropatia diabética, mostrado pelo aumento de superóxido, aumento da expressão gênica de subunidades do complexo NADPH oxidase e detecção *in situ* de ROS e NO [44].

O NO também representa um composto redox vasoativo importante [45] atuando tanto intra ou extracelularmente, como intermediário de funções no endotélio vascular, regulando a hemostasia, a permeseletividade endotelial e tônus vascular [12]. Este gás é sintetizado a partir da oxidação de um dos dois nitrogênios guanidino da L-arginina, que é convertida em L-citrulina [46]. Isoformas de NOS foram classificadas em duas categorias: constitutivas (c-NOS) e induzíveis (i-NOS). As c-NOS produzem pequenas quantidades de NO e sua ativação depende da interação com a calmodulina, que, por sua vez, é controlada pelos níveis de cálcio. A i-NOS não é muito expressa sob condições normais, mas é induzida por citocinas e/ou endotoxinas em células incluindo as endoteliais [46]. O endotélio normal é caracterizado por um funcionamento apropriado da NOS endotelial (e-NOS), uma forma de c-NOS, que está constantemente ativada e produz poucas quantidades de NO. Contudo, quando desacoplada de seus substratos normais, a e-NOS pode produzir grandes quantidades de anion superóxido [47], concomitantemente a uma diminuição de produção de NO.

Bioquimicamente, o NO atua através da interação com proteínas contendo grupos sulfidril, centros Fe-S, grupos heme e ROS [48]. O aumento exacerbado da produção de NO e íons superóxido, particularmente em sítios de inflamação pelas oxidases correspondentes, favorece a formação do peroxinitrito (Reação 1), um poderoso oxidante de biomoléculas, especialmente de resíduos tirosina, além de diminuir a biodisponibilidade do NO (Reação 1) [49, 50].

$$^{\bullet}NO + O_2^{\bullet -} \Rightarrow ONOO^{-} \qquad \text{Reação 1}$$

Estudos em pacientes mostraram que a DRC altera a liberação de NO, a expressão e atividade das NOS e a nitração de proteínas [51-54], bem como estudos *in vitro* com células endoteliais [55] e estudos com modelos animais [45]. Todos estes estudos comprovam a ação do meio urêmico na modificação da liberação de NO e na expressão das isoformas de NOS. Examinado criticamente, veremos que a liberação de NO pode estar aumentada, mas a bioatividade diminuída [56].

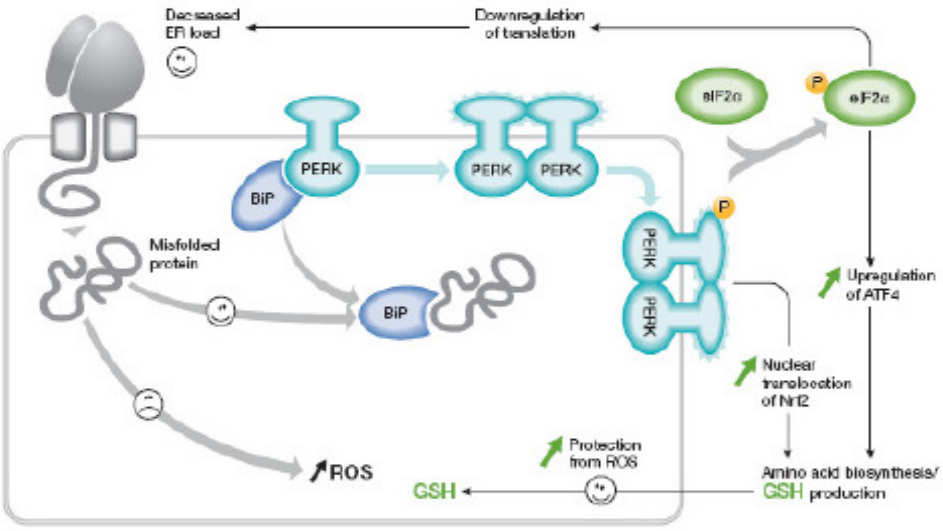
Tratamentos com drogas que combatem o estresse oxidativo têm sido usados em pacientes com DRC como tentativa para melhorar o risco de doença cardiovascular nestes pacientes. Estudos com modelos animais, camundongos *knockout* para ApoE com e sem DRC, mostraram que o tratamento com drogas com efeitos antioxidantes inibem a progressão da formação da lesão aterosclerótica e também favorece em sua regressão [57], bem como a diminuição do estresse nitrosativo [58]. Num estudo randomizado, Tepel e col mostraram que o uso do antioxidante N-acetilcisteina (NAC) reduz os eventos cardiovasculares em pacientes com DRC em estágio final [59]. E em um estudo sobre o efeito da Vitamina D, Varizi e colaboradores mostraram a diminuição da formação das proteínas nitradas e o aumento da expressão de NOS nos pacientes DRC [51]. Assim, intervenções favorecendo a redução de ROS com antioxidantes, provenientes da dieta ou medicamentos, parecem atenuar ou prevenir o estresse oxidativo e conseqüentemente, os eventos cardiovasculares na DRC, através de mecanismos ainda não tão bem esclarecidos.

1.2.2. Estresse do Reticulo Endoplasmático

Outra mudança fenotípica relacionada ao estresse oxidativo é o chamado "estresse do retículo endoplasmático". O reticulo endoplasmático (RE) é o principal local para o dobramento e maturação de proteínas. Perturbações que alteram sua homeostase podem levar a uma acumulação de proteínas mal-dobradas, chamado estresse do RE, podendo ser uma ameaça às células [60]. A resposta ao estresse do retículo em células de mamíferos, levam a ativação de quinases transmembrânicas, a quinase e endoribonuclease IRE1 e a quinase *PKR-like ER kinase* (PERK) e o fator de ativação de transcrição transmembrânico 6 (ATF6). A ativação dos três componentes da resposta às proteínas mal dobradas depende da dissociação de uma chaperona chamada Binding Proteins (BiP) (GRP78). Em células não estressadas BiP retém o fator de transcrição ATF6 no lúmem do RE e também está associada com o domínio lumenal de IRE1 e PERK. Sob estresse, BiP é competitivamente retirada do domínio lumenal de

IRE1 e PERK e de ATF6 pelo excesso de proteínas não dobradas, por terem preferência na ligação com BiP. A ativação de PERK, ATF6 e IRE1 têm como resultado a atenuação da tradução de novas proteínas e ativação de tradução de proteínas específicas de degradação e chaperonas, como a própria BiP, que tem a seqüência KDEL em sua seqüência de aminoácidos [60-63]. A tradução do fator de transcrição ATF4 é estimulada e, com a ativação de Nrf2 leva a indução de genes envolvidos na síntese de GSH. O nível celular de glutationa é aumentado, o qual pode potencialmente proteger a célula de danos causados pelas espécies reativas, produzidas durante o estresse do RE [61].

Figura 2: Associação do estresse do retículo endoplasmático com o estresse oxidativo



Fonte: Chakravarthi, 2006 [61]

O estresse do RE é uma resposta celular, que quando ocorre por tempo prolongado pode resultar em morte celular por apoptose e contribuir para patogênese de várias doenças, como Alzheimer, Parkinson e diabetes [63]. Há estudos mostrando que a hiper-homocisteinemia induz apoptose em células endoteliais através da resposta ao estresse do RE [15]. Dickhout e colaboradores mostraram que o peroxinitrito induz estresse do RE em lesões arterioscleróticas [64].

1.2.3 Calcificação vascular

O padrão da doença vascular na população DRC difere da população em geral. A doença vascular tradicional compreende placas ricas em lipídios produzindo estenose focal e potencial ruptura da placa e subseqüente trombose. Na DRC essa placa é caracterizada por intensa calcificação mesmo em fase inicial [65-68]. Estudos demonstraram que calcificação coronária é detectável em grandes proporções em pacientes jovens [68, 69]. A calcificação ocorre na camada íntima, e principalmente na media, e é um marcador da aterosclerose acelerada, observada na uremia [5, 65].

A calcificação na íntima tem sido relacionada a fatores de risco tradicionais, enquanto que na média correlaciona-se com desbalanço Ca x P e menor idade [70]. O cálcio e o fósforo existem no soro em um balanço refinado, ditado por simples química: o conceito de solubilidade significa que mesmo pequenos aumentos na concentração de fosfato sérico resultam no aumento da precipitação de fosfato de cálcio. O tecido mais exposto ao soro, o tecido vascular, é o mais afetado, mas a preponderância na calcificação da média ainda não está clara. A precipitação de fosfato de cálcio resulta na estimulação do hormônio paratireoideano (PTH), para manutenção do cálcio sérico. PTH é também estimulado pelo nível sérico de fosfato [65, 71]. Também, a calcificação como conseqüência do fator de risco não tradicional em DRC, o desbalanço Ca X P, e sua relação com estresse oxidativo merece maior atenção [72].

1.3 Justificativa

O fato da terapia antioxidante, principalmente a NAC, ter demonstrado uma inibição do risco cardiovascular em doentes renais crônicos [59], em contraste com a ineficácia na população geral, tem motivado a investigação dos processos fisiopatológicos da DRC que justifiquem tal resultado. Neste contexto, esclarecer o papel do estresse oxidativo vascular na fisiopatologia da DRC poderia contribuir para a busca de tratamentos mais eficazes e mais econômicos que diminuíssem a mortalidade destes pacientes.

Nesse estudo pretendemos demonstrar através de abordagens *in vivo* e *in vitro* que o ambiente urêmico pode desencadear uma resposta oxidativa no endotélio, contribuindo para a aceleração da aterosclerose observada nesses pacientes.

2. OBJETIVOS

O objetivo principal desse projeto foi analisar efeitos redox induzidos pelo acúmulo de toxinas urêmicas sobre tecidos vasculares e avaliar o efeito do antioxidante NAC. Particularmente:

(i) analisar a ocorrência e a correlação de estresse nitrativo e da calcificação em artérias de pacientes em hemodiálise e voluntários saudáveis, sem DRC;

(ii) avaliar o estresse nitroxidativo em células endoteliais e a capacidade do antioxidante NAC em inibir tal estresse.

3. ARTIGO "INCREASED CALCIFICATION AND PROTEIN NITRATION IN ARTERIES OF CHRONIC KIDNEY DISEASE PATIENTS".

INCREASED CALCIFICATION AND PROTEIN NITRATION IN ARTERIES OF CHRONIC KIDNEY DISEASE PATIENTS

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Abstract

Background: Cardiovascular disease (CVD) is frequent complication in chronic kidney disease (CKD). Classic risk factors for the development of CVD are predominant in CKD patients, but CKD-related risk factors, including disturbances of mineral metabolism and oxidative stress, have been proposed to play a pivotal role in this increased risk. The aim of this study was analyze vascular calcification and tyrosine nitration from CKD patients submitted to renal transplant when compared with their donors. **Methods:** External iliac and renal arteries segments from 16 stage 5 CKD patients and 13 donor controls, respectively, were collected during the transplantation procedure. Histological analysis was based on Stary classification, calcification was assessed by alizarin staining and nitrotyrosine production was analyzed by immunohistochemistry. **Results:** Patients of CKD group presented a higher prevalence of lesions type Stary 2 and 3, while the prevalence of lesions in control patients group was type 1 (Chi^2=11.2; p<0.05). Additionally, the Starry score was significantly higher in the CKD patients group (2.3±0.2) when compared to the control group (1.5±0.2; p<0.05). Vascular calcifications were observed in 68% of CKD patients, compared with 7% in the control group (chi^2 =12.4; p<0.0005). There was a significant correlation between serum calcium and the score of medium calcification (R=0.49; p=0.05), the correlation of serum phosphorus and the score of calcium in the intima (R=0.57; p<0.05), media-intima (R=0.58; p<0.05) and media (R=0.58; p<0.05), and finally between the Ca x P product and the intima (R=0.60), media-intima (R=0.61; p<0.01) and media (R=0.65; p<0.005) calcium scores. Patients with vascular calcification presented older age, more accentuated Ca and P metabolism alterations, anemia and dyslipidemia. Immunohistochemistry revealed a significant increase in nitrotyrosine staining in arteries from CDK patients (62,179±4,264) compared with control donnors (20,208±3,621; p<0.0001). **Conclusion:** The arteriopathy in the CKD patients appears in an early age and seems to be distinct of the arteriopathy of the general population, specially due to intense calcification and vascular oxidative stress, in addition to its correlation with predictors of mortality, such as dyslipidemia, anemia and mineral metabolism disorders.

Keywords: Atherosclerosis, chronic kidney disease, vascular calcification, nitrotyrosine, cardiovascular disease.

Introduction

Cardiovascular diseases (CVD) represent the most important cause of death in patients with chronic kidney disease (CKD), whose mortality risk is manifold higher when compared to the general population [1]. The reasons for this strikingly increased risk are still unknown, and the mechanisms by which the CVD develops in such an accelerated fashion remain to be unveiled [2]. Several classic risk factors for the development of atherosclerotic CVD, i.e. diabetes, hypertension, dyslipidemia and obesity are predominant in CKD patients, but cannot fully explain the CVD burden in this population. Thus, CKD-related risk factors, including disturbances of mineral metabolism, anemia, oxidative stress and inflammation, have been proposed to play a pivotal role in this increased risk [3]. Although the mechanistic involvement of these risk factors in the CKD-related CVD have been clearly supported by experimental studies and also linked to increased mortality in CKD, few clinical studies have shown that they are directly involved in the physiopathology of the CVD.

The profile of the vascular disease in the CKD population differs from the general population. Traditional vascular disease comprises intimal disease with lipid-rich plaques producing focal stenoses and the potential for plaque rupture and subsequent thrombosis [4]. Media alterations, including smooth muscle cell proliferation and calcification are features of elderly patients and diabetics [4]. On the other hand, the arteriopathy observed in experimental models of CKD (CKD induced in the Apo E knockout mice) show that plaque formation is accelerated and magnified, and both intima and media are characterized by intense calcification [5]. In the same model, signs of vascular inflammation and oxidative stress, represented by the increased expression of adhesion molecules and generation of vascular oxidative stress are observed [6].

Due to the difficulties in accessing arteries from CKD patients, few clinical studies described the vascular changes in humans, particularly in assymptomatic patients [7]. Renal transplantation offers an interesting opportunity to access vascular tissue of assymptomatic CKD patients (and healthy controls – the donors) without exposing patients to risk. We hypothesize that signs of accelerated atherosclerosis are present in the arteries of patients with CKD, even in the early and asymptomatic phases of the disease and are associated with markers of increased CVD risk. Thus, the aim of the present study was to histologically analyze calcification degree and nitrative stress, reflecting an increased formation of reactive

oxygen and nitrogen species, in arteries of CKD patients and to compare it with a group of healthy controls.

Subjects and Methods

Patients in stage 5 CKD who accepted to participate in the study and underwent a living donor renal transplantation were included in the study. Their kidney donors served as the control group. Exclusion criteria included smoking, diabetes, systemic inflammatory disease and the history or symptoms of CVD prior to the transplantation (both groups underwent a screening of CVD within 3 months of the study). Groups were matched for age, lipid levels, body mass index and gender distribution. The institution's ethics committee approved the protocol, and all patients signed an informed consent.

At the moment of the transplantation, external iliac and renal arteries segments were collected from CKD patients and controls, respectively, and were immediately fixed in 5 mL of 10% formalin. After an overnight period, specimens were embedded in paraffin and 5 µm sections were prepared. Atherosclerotic lesions were classified according the Stary's classification [8, 9] (Figure 1), based on intimal thickness, presence of foam cells, lipid, and atheroma in HE-counterstained sections. For evaluation of calcification, Alizarin red (pH of 4.2) staining was employed, which stains reddish-brown to calcium carbonate and phosphate (Figure 2) [10].

For the immunohistochemistry, endogenous peroxidase was blocked with 1% H_2O_2 and unespecific sites with 1% BSA during 45 minutes. Nitrotyrosine was detected using a mouse monoclonal anti-nitrotyrosine antibody (1:300, Sigma), followed by incubation with the LSAB kit (DAKO). Reaction was developed with DAB and cells were counterstained with hematoxylin. As a control, the primary antibody was omitted, resulting in negative staining. Images were captured by a microscope and the brownish density was quantified using an image analysis software (Image ProPlus).

Results were analyzed using JMP 7.0 statistical package. The classification values (au) were considered as continuous variable with *t*-Student as a comparative test. The correlation was made using the Pearson test and prevalence analysis with Chi square test (Chi^2). Results from the experiments are expressed as mean ± standard deviation.

Results

During the period from September 2005 and January 2006, 16 CKD patients and their kidney donors were recruited in the study. The main characteristics of the study population are described in Table 1. The major causes of CKD in such patients were hypertensive nephrosclerosis in 37% and chronic glomerulonephritis in 25% of patients. Most patients (81%) were treated with hemodialysis before the transplantation and the remaining were on peritoneal dialysis. The mean time on renal replacement therapy was 33±8 months.

Regarding the atherosclerotic findings evaluated with the Stary's classification, we observed that CKD patients presented a significantly higher degree of lesion, represented by scores 2 and 3, while lesions score 1 predominated in the control group (Chi^2=11.2; p<0.05) (Figure 1). Therefore, the mean Stary score in the CKD group was significantly higher (2.3±0.2) when compared to the control group (1.5±0.2; p<0.05) (Figure 2). When CKD patients were subdivided according to the atherosclerosis score (Stary I e II versus Stary ≥ 3), we observed that only BMI was significantly different between the groups (Table 2).

In a large proportion (68%) of CKD patients we observed vascular calcifications, which were diffusely located in the intima layer, intima-media transition and more intensively in the media layer. In contrast, only one patient of the control group presented calcification that was restricted to the media layer (chi^2 =12.4; p<0.0005). Aging, female sex, high serum phosphorus concentrations, high calcium / phosphorus product, low hematocrit and pronounced dyslipidemia predisposed to an increased vascular calcification (Table 3).

The group with vascular calcification was older, showed a higher proportion of females, presented higher serum phosphorus, higher calcium / phosphorus product, lower hematocrit and more pronounced dislipidemia (Table 3). There was a positive correlation between serum calcium and the media layer calcification score (R=0.49; p=0.05); between serum phosphorous and intima (R=0.57; p<0.05), media-intima (R=0.58; p<0.05) and media (R=0.58; p<0.05) calcification scores and finally between the calcium / phosphorus product and intima (R=0.60), media-intima (R=0.61; p<0.01) and media (R=0.65; p<0.005) calcium scores. A significant bordeline correlation was observed between media calcification and age (R=0.49; p=0.06).

Immunohistochemistry for nitrotyrosine revealed a significant increase in nitrotyrosine production in arteries from CDK patients (62,179 ± 4,264) compared with control donors

(20,208 ± 3,621; $p<0.0001$) (Figure 4). In addition, nitrotyrosine staining was significantly stronger in arteries with media calcification (61,556 ± 6,072) versus arteries without media calcification (29,163 ± 5,760; $p<0.005$) (Figure 5). The same association was observed with intima calcification (65,894 ± 5,617 vs. 28,953 ± 4,790; $p<0.0001$) and medio-intima calcification (66,804 ± 6,547 vs 31,500 ± 5,000; $p<0.0005$). A positive correlation between calcification scores in medio-intima and nitrotyrosine staining was observed ($R=0.63$; $p<0.05$) in CDK patients.

Discussion

Although the accelerated atherosclerosis and the increased CVD risk in patients with CKD are well recognized, the mechanisms behind these observations are still to be clearly defined in human studies. In the present study, we report that the CKD alterations observed in arteries of an asymptomatic group of patients are characterized by accelerated atherosclerosis, intense and diffuse vascular calcification and signs of vascular oxidative stress. In addition, histological findings of calcification were significantly associated with traditional (dyslipidemia, hypertension) and non-traditional (anemia and mineral metabolism disorders and vascular oxidative stress) risk factors for CVD mortality.

The vascular disease process begins with endothelial dysfunction, which can be triggered by many factors, such as ageing, dyslipidemia, hypertension and smoking [9]. The endothelial aggression leads to endothelial expression of molecules that are involved in cytokine-mediated leukocyte adhesion. The macrophage phagocytes local modified lipids (primarily oxidized LDL) forming the precursor of the atherosclerotic plaque, the foam cells. As a consequence of the additional insult, the macrophage, through chemokines and adhesion molecules, recruit more leukocytes to adhere on the plaque surface leading to the development of atherosclerosis [9].

In CKD patients, additional uremia-related factors that induce endothelial dysfunction, vascular inflammation and oxidative stress are potentially important in the development of a peculiar form of vascular disease related to the presence of CKD [11]. In animal models of CKD-related CVD, a consistent observation is that the presence of CKD (even when renal dysfunction is mild) accelerates and amplifies plaque formation [5]. Our study confirms this

observation, since patients with CKD patients presented significantly higher atherosclerotic scores in comparison to their controls, who were matched for the most important traditional risk factors for CVD, namely smoking, age, gender and dyslipidemia. It is important to point out that both patients and controls were investigated and proved negative to ischemic heart disease, defining an early stage of the atherosclerotic process. Interestingly, when we compared CKD patients with high atherosclerotic scores to the one with low scores, the only significant observation was that patients with more striking signs of atherosclerotic CVD presented lower body index. Indeed, although some risk factors such as hypertension and dyslipidemia are not clearly related to patient outcome, low body mass index has been consistently associated with high mortality in dialysis patients, most likely reflecting malnutrition and increased inflammatory activity (MIA syndrome) [2]. Since increased expression of adhesion molecules is an important observation in the CKD-related CVD animal model, our results may indicate that malnutrition and inflammation could be also involved in the acceleration of the atherosclerotic process in humans through enhanced vascular inflammation. The exact mechanism mediating the relationship between malnutrition, inflammation and atherosclerosis, however, remains to be investigated. Also, studies with larger number of patients will help to define which factors are related to the increase in plaque formation and size in the CKD population.

Although accelerated plaque formation is an important finding both in clinical and experimental studies, the most striking (and peculiar) characteristic of CKD-related CVD is vascular calcification. It is well established that abnormalities in mineral metabolism appear early in the course of CKD and result in clinically relevant consequences, particularly linked to high cardiovascular morbidity and mortality [12]. There is an increasing body of evidence that supports the thesis that elevated serum levels of phosphorus and calcium and deficiency of inhibitors of calcification are important factors in the progression of vascular calcification in patients with end-stage renal disease [12]. In our study, we observed that the majority of the CKD patients presented vascular calcification, despite the fact that the population was very young, asymptomatic and presented a low prevalence of risk factors for CVD. Older patients presented higher calcification scores, what is consistent with studies that utilized non invasive methods [1, 12]. Interestingly, there were more calcified arteries in patients with the primary diagnosis of hypertension related kidney disease, suggesting the role of hypertension in the calcification process. In addition, patients with signs of vascular calcification showed

clear evidence of disturbances of mineral metabolism, namely higher serum phosphorus leve and calcium / phosphorus product, demonstrating directly in the artery that the increased ris of CVD mortality linked to those elements may be related to increased vascular calcificatio Finally, the observation that patients with calcification presented higher cholesterol levels ar lower hematocrit points to the need to identify alternative pathogenetic mechanisms that ma be important causes of the high calcification burden observed in CKD.

Oxidative stress is increasingly being suggested to play a central role in th pathogenesis of cardiovascular disease in CKD. Increased production of nitric oxide and/ superoxide-derived oxidants leads to an oxidative stress condition. All cells of th atherosclerotic lesions, namely endothelial cells, macrophages, and smooth muscle cells, a able to actively participate in the generation of such species [13, 14]. Nitrotyrosine, which ha been considered as a novel independent marker of cardiovascular disease [14-18]. In vivo, is produced non-enzymatically, via peroxynitrite and/or enzymatically, via heme-peroxidas dependent nitration [18]. Our results describe for the first time an increased protein nitratio in CKD patients arteries, in agreement with the experimental models findings[19-21 although we are unable to distinguish between the two production routes at the momen Moreover, a clear association between protein nitration and signs of vascular calcification this population is shown.

In conclusion, this study represents an *in vivo* evidence that the CKD arteriopath emerges prematurely and seems to be distinct of CVD observed in the general populatio mainly due to intense calcification and signs of vascular oxidative stress. Vascul calcification correlated with dyslipidemia, anemia, mineral metabolism alterations, and oxidative stress. The investigation of the calcification and oxidative stress pathways can lea to a better understanding of the mechanisms of the CKD-related CVD and more effecti preventive and therapeutic strategies to reduce morbid-mortality in this high risk population.

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Table 1: Main characteristics of the study control group and patients with chronic kidney disease in the evaluation performed within 3 months of the transplant. All patients and controls presented negative screening for ischemic heart disease.

	Control Group	CKD group	*p* value
Age (years)	35±5	36±13	ns
Males (%)	58	56	ns
Body mass index	24±3	24±3	ns
Fasting blood glucose (mg%)	94	96	ns
LDL cholesterol (mg%)	116±12	119±17	ns
HDL cholesterol (mg%)	46±10	44±9	ns
Creatinine (mg%)	0.8±0.2	5.6±2.1	<0.0001

ns: not significant

Table 2: Atherosclerosis classification in CKD patients. Correlation with clinical a laboratorial parameters, according to the atherosclerotic lesion severity (Stary I e II vers Stary ≥ 3).

	Stary I e II	**Stary ≥III**	***p* value**
N	7	9	
Age (years)	39±5	33±4	ns
Males (%)	100	45	ns
Dialysis time (months)	26±15	38±13	ns
Hemodialysis (%)	85	77	ns
Body mass index	25±1	22±1	0.05
Nephrosclerosis (%)	22	57	ns
Hematocrit (%)	32±1	31±1	ns
Glicemia (mg/dL)	104±11	132±10	ns
Calcium (mg/dL)	9.3±0.4	9.1±1	ns
Phosphorous (mg/dL)	5.3±1.1	5.5 ±0.9	ns
Ca x P product	49± 9	50±8	ns
PTH (UI)	207±94	279±79	ns
Total cholesterol (mg/dL)	197±19	169±16	ns
LDL (mg/dL)	115±12	108±10	ns
HDL (mg/dL)	49±6	40±5	ns

Table 3: Calcification classification in CKD patients: Correlation with clinical and laboratorial parameters, according to the atherosclerotic calcification

	no calcification	**calcification**	***p* value**
N	5	11	
Age (years)	27±5	41±3	0.04
Male sex (%)	100	45	0.01
Dialysis time (months)	48±17	26±11	ns
Homodialysis (%)	60	91	ns
Body mass index	22±1	24±1	ns
Nephrosclerosis (%)	0	54	0.01
Hematocrit (%)	35±1	30±1	0.02
Glicemia (mg/dL)	137±14	112±9	ns
Calcium (mg/dL)	8.7±0,4	9.4±0.3	ns
Phosphorous (mg/dL)	3.2±1.1	6.4±0.7	0.03
Ca x P product	29±9	59±6	0.01
PTH (UI)	252±64	220±214	ns
Total Cholesterol (mg/dL)	133±20	197±12	0.02
LDL (mg/dL)	84±12	121±7	0.005
HDL (mg/dL)	38±8	46±4	ns

Figure 1: Representative figures of artherosclerotic lesions according to the Stary´s classification (stained using eosin/hematoxylin). Type I (A), with integrity of internal elast and type IV (B), representing foam cells and pool of extracellular lipids (magnification 400X).

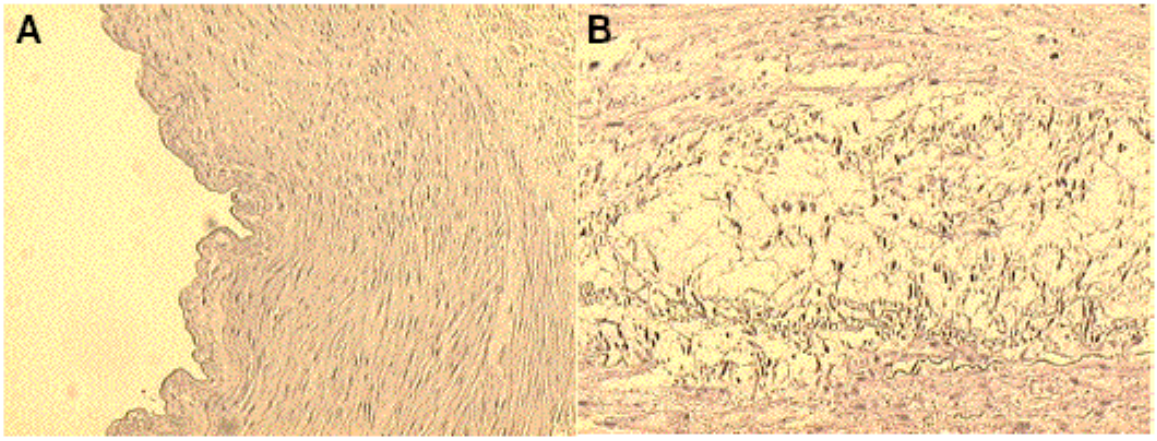


Figure 2: Comparison between Stary scores in arteries of CKD patients and controls. (*p<0.05)

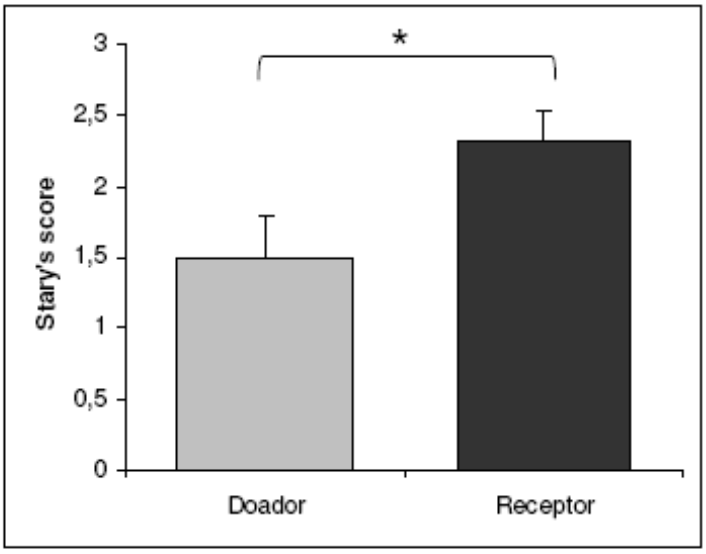


Figure 3: Representative images of calcification (alizarin red (pH 4.2), which stains reddish-brown to calcium carbonate and phosphate). Figures show plaque (A) and diffuse (B) calcification in arteries of patients with chronic kidney disease (magnification 400X).

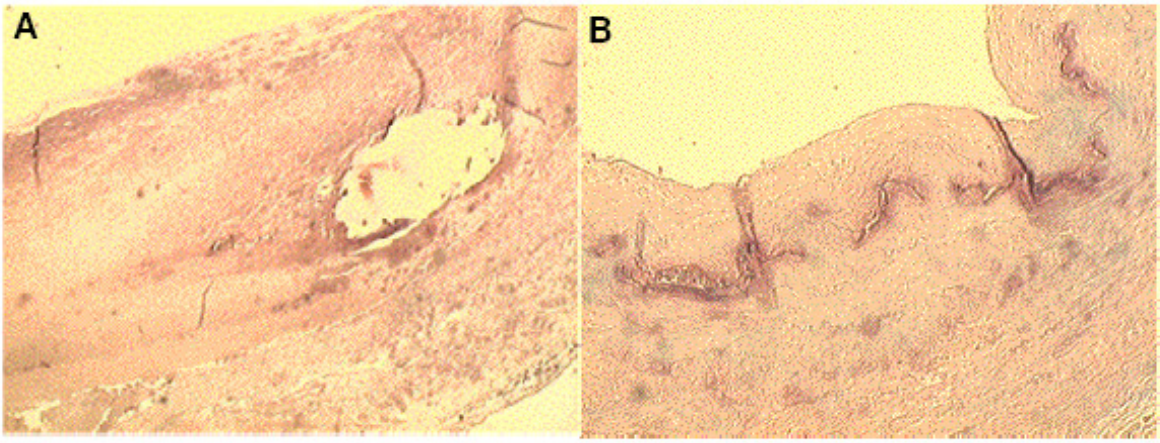


Figure 4: Representative images of arteries immunostained with anti-nitrotyrosine. Staining was quantified using image analysis software (*p<0.0001) (A). Representative nitrotyrosine immunostaining of arteries from healthy donor (B) and chronic kidney disease patient (C) (magnification 400X).

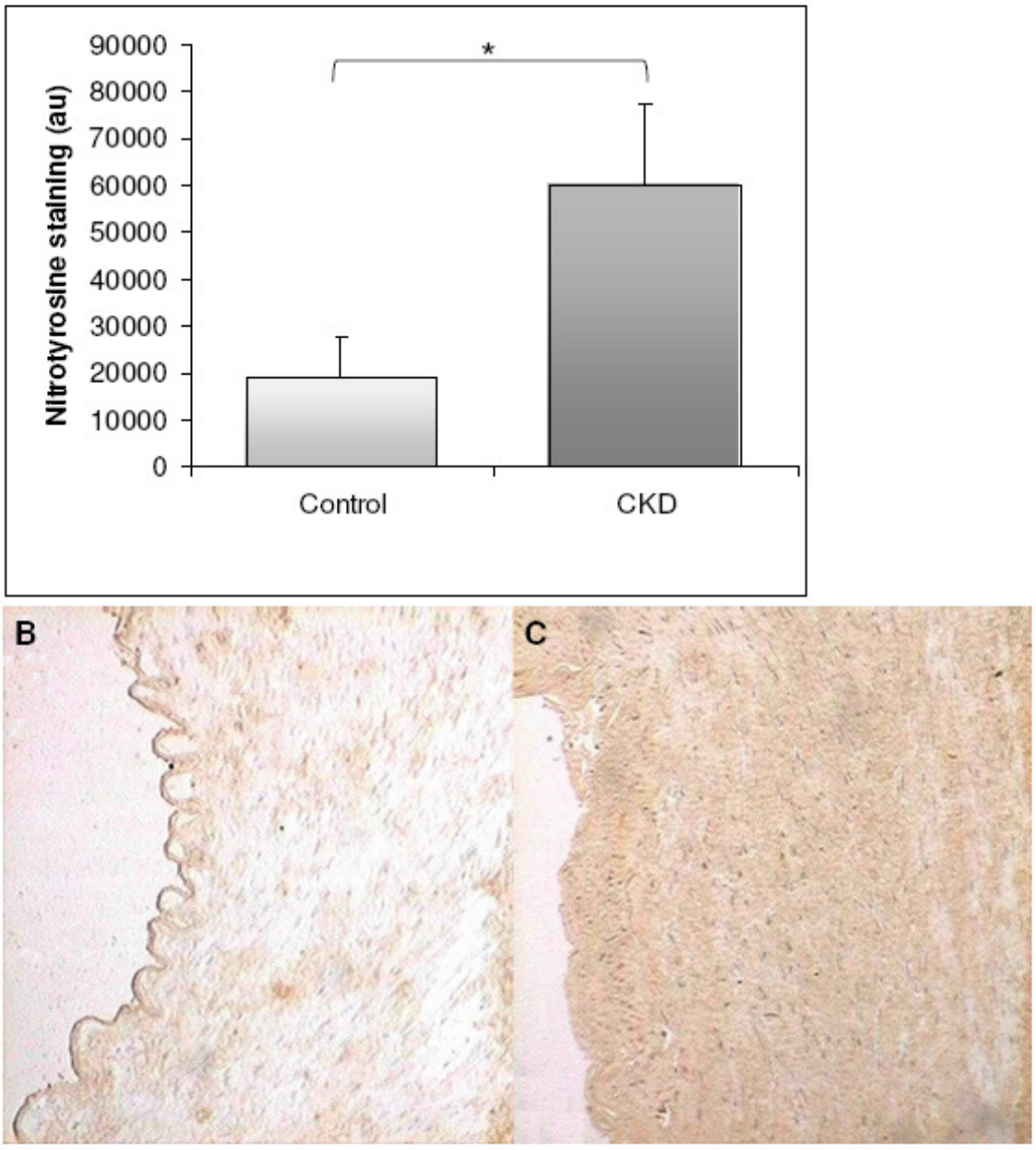
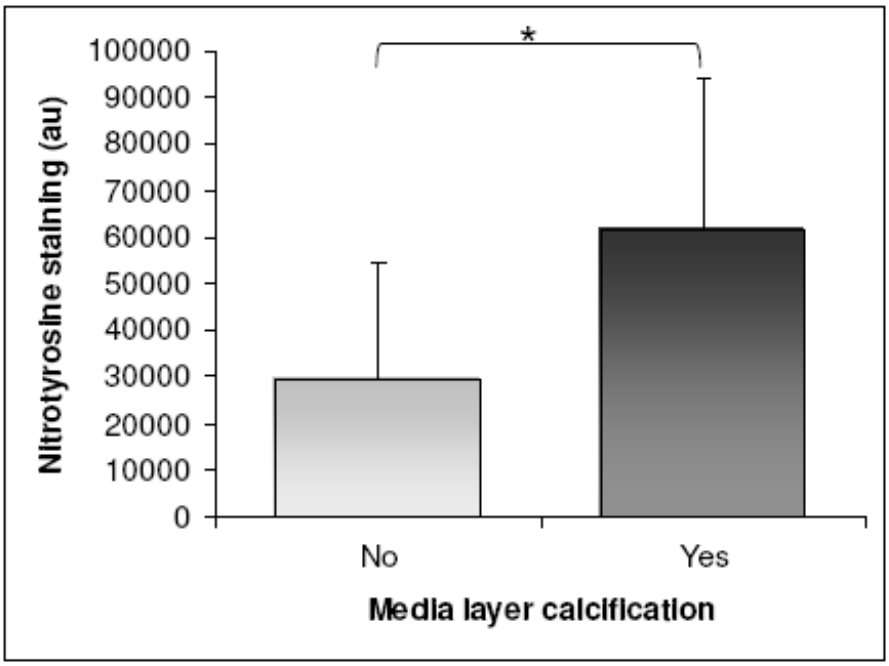


Figure 5: Association between the presence of calcification and nitrotyrosine staining in arteries of patients with chronic kidney disease (*p<0.005).



4. ARTIGO "UREMIC SERUM INDUCES BOTH NITROXIDATIVE AND ER STRESSES IN ENDOTHELIAL CELLS"

UREMIC SERUM INDUCES BOTH NITROXIDATIVE AND ER STRESSES IN ENDOTHELIAL CELLS

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ABSTRACT

Chronic kidney disease (CKD) patients are at greater risk of developing cardiovascular diseases (CVD) than patients with normal kidney function. Among factors contributing to vascular pathologies, inflammation and oxidative stress have been considered as non traditional risk factors. Uremia, peculiar solely to the CKD population, may represent a novel factor. Since CKD is a complex disease, here we analyzed the redox effects of uremia *per se* in endothelial cells (EC) in culture. Our results show that incubation of EC to 10% uremic serum increases NADPH oxidase activity and decreases nitric oxide bioavailability, resulting in increased nitrotyrosine production and ER stress induction compared to non uremic serum treatment. In addition, N-acetylcysteine (NAC) inhibits superoxide formation and ER stress, probably through inhibiting NADPH oxidase and peroxynitrite production. These results provide some molecular basis for the mechanisms underlying endothelial dysfunction in CKD patients and for the observed clinical benefits of NAC in renal diseases.

Keywords: uremia, oxidative stress, nitric oxide, superoxide, endothelial cells

Footnotes: DHE: dihydroethydium, DAF: diaminofluorescein diacetate, ROS: reactive oxygen species, RNS: reactive nitrogen species, DPI: diphenyleneiodonium, EC: endothelial cell, NAC: N-acetycysteine, CKD: chronic kidney disease, CVD: cardiovascular disease, GSH: glutathione, GSSG: oxidized glutathione, ER: endoplasmic reticulum, NO: nitric oxide, SOD: superoxide dismutase

INTRODUCTION

Chronic kidney disease (CKD) patients are at greater risk of developing atherosclerosis than patients with normal kidney function [1]. These patients, especially those receiving maintenance hemodialysis therapy (HD), present a chronic inflammatory state [2] and an increased oxidative stress [3, 4], which, in combination seems to be a major contributor to cardiovascular disease [1, 2]. Moreover, oxidative stress biomarkers are correlated with uremic toxins levels [5]. Indeed, several works have demonstrated that oxidative stress is evident in CKD patients. Increases in lipid peroxidation, advanced oxidation protein products and alterations in glutathione (GSH) system are the most popular oxidative biomarkers measured in blood samples [6-8]. Blood cells are also subject to oxidative stress in CKD populations. Decrease on GSH associated with a reduced GSH peroxidase and GSSG reductase activities leukocytes [9] and red blood cells [10] from CKD patients.

Recently, the participation of uremic toxins in vascular injury has been considered. Incubation of polymorphonuclear cells with uremic plasma increases apoptosis [11], similar to the in vivo finding [12] . Smooth muscle cells are also sensitive to the toxic effects of uremic serum, increasing the expression of calcification proteins, such as osteopontin [13] and bone matrix proteins [14]. Finally, some works have reported that endothelial cells cultivated in the presence of uremic serum increase the proliferation rate[15] and increases the expression of pro-inflammatory genes [16].

Oxidative stress is also associated to phenotypic process, including endoplasmic reticulum (ER) stress [17]. The ER is a principal site for biosynthesis of proteins, steroids, cholesterol and other lipids [18]. Conditions where expression of mutant, folding-incompetent proteins are increased, or levels of reducing agents are increased trigger an ER stress response. This response triggers the expressor of ER protein, such chaperone GRP78 and GRP94 [19].

Here we show that incubation of EC with uremic serum induces an increased superoxide formation, resulting in increased nitrotyrosine production and ER stress induction, events inhibitable by a single exposure to NAC. Our results, as far as we know, is the first to characterize the endothelial cell redox response to uremic serum.

RESULTS

Uremic and non uremic sera pool characterization

Sera pools were biochemically analyzed to characterize their uremic and redox state. Results showed that lipidic and glycemic profiles were normal and similar between the two pools (Table 1). Increased phosphorus, urea, uric acid and creatinine levels were observed in the uremic pool. Although acute inflammation marker C-reactive protein (CRP) concentration was high in such pool, the non-uremic pool also presented a slightly elevated CRP level. These values are likely due to the fact that, except for DM, other inflammatory diseases, such as cardiovascular disease (CVD), were not excluded in our populations.

In addition, we observed differences in total thiol concentrations between non-uremic (1620 μM) and uremic (580 μM) sera pools. Oxidative stress was still confirmed by carbonylated (0.0134 *vs.* 0.0192 nmol/mg albumin), and nitrated (20 *vs.* 26 au/μg protein) protein levels (Table 1), as expected [20, 21]. These data confirm the uremic syndrome and show an altered redox balance in HD patient blood.

Uremic serum stimulates $O_2^{\bullet-}$ production by EC membrane fractions

DHE-derived fluorescence of membrane fractions from EC treated with uremic pool was significantly higher that that from the non uremic treatment (Figure 1), probably reflecting an increased $O_2^{\bullet-}$ production [22]. Superoxide-mediated DHE oxidation was demonstrated by an intense inhibition of the fluorescence by the presence of SOD in the reaction medium. Cellular sources of superoxide anions include the mitochondrial respiratory chain, uncoupled NOS and cytosolic oxidases [23]. In the vasculature, however, NAD(P)H oxidase seems to be the main $O_2^{\bullet-}$ source, acting as a central key in several redox signaling pathways [24]. The endothelial NAD(P)H oxidase complex has been recently characterized [23, 25]. Indeed, membrane fractions from 100 nM angiotensin II-treated EC produced an intense fluorescence (2.81 ± 0.86 fold over negative control, data not shown). To investigate whether superoxide was a product of the NAD(P)H oxidase activation, DPI, a flavoenzyme inhibitor, was employed. In this case, DHE oxidation was diminished in both treatments, indicating that NADPH oxidase was activated by uremic serum (Figure 1). When EC were previously exposed

to the antioxidant NAC, DHE-increased oxidation stimulated by uremic treatment was inhibited by 60%, whereas no effect was observed in non-uremic treatment (Figure 1).

Uremic serum disturbs NO pathways in EC

Nitric oxide and nitric oxide derived species were detected in whole cells by flow citometry, using diaminofluorescein diacetate (DAF). Cells treated with LPS (2 µg/mL, 24h) produced a significantly increased mean fluorescence (11.39 ± 1.82) compared to control cells (3.54 ± 0.59) (data not shown), indicating that the method was responding to NO level variations. Therefore, the potential effect of uremic serum on NO availability in EC was analyzed. Incubation of EC with uremic serum for 3 hours did not modify the NO release compared with non uremic serum (Figure 2). After 24 hours, fluorescence induced by non-uremic serum increased, while that induced by uremic pool remained unchanged (Figure 2). NAC pre-treatment prevented DAF nitrosation stimulated by non uremic exposure, but had no effect in uremic treatment after 24h (Figure 2).

To investigate if the altered NO levels were a consequence of a change in inducible NO synthase (iNOS) expression, a western blot assay was performed to quantify this isoform. Inducible NOS expression was increased after LPS stimulation (data not shown). However, it was not altered either after 3 or 24 hours incubation with non-uremic pool and uremic pools (Figure 3). There was no significant difference in iNOS expression when EC were pre-exposed to NAC (Figure 2).

Nitric oxide is a precursor of more oxidant nitrogen reactive species, which can, among other reactions, nitrate tyrosine residues [26]. Therefore, nitrotyrosine level was used as an index of increased production of reactive nitrogen and oxygen species. Dot blot assays with anti-3-nitrotyrosine showed that after 3 hours protein nitration levels were similar between the two groups (Figure 4). After 24-hour treatment with uremic pool, however, EC showed a significantly increased 3-nitrotyrosine production compared to non-uremic control pool treatment. Also, NAC pre-treatment did not significantly modify this pattern (Figure 4).

Uremic serum induces ER stress in EC

Recently, it has been demonstrated that an increased cellular oxidative status can converge into an ER stress [27]. Therefore, we assessed the ER stress response by analyzing the expression of endoplasmic reticulum molecular chaperone GRP78 and

GRP94 with anti-KDEL antibody. Tunicamycin (5μg/mL) was used as a positive control, and it induced an increased GRP78 protein levels when compared to negative control (1.36 ± 0.25 GRP78/β-actin ratio *vs.* 0.94 ± 0.20 GRP78/β-actin ratio, $p<0.05$, data not showed). GRP 94 expression was enhanced by uremic serum, while no significant difference was observed for GRP78 after 3h with non uremic sera pool. However, 24h-treatment with uremic serum induced an increased expression of both GRP94 and GRP78 proteins expression when compared with non-uremic control treatment. This increase was inhibited by pre-treatment with NAC (Figure 5B).

DISCUSSION

Overproduction of reactive oxygen and nitrogen species has long been considered a CVD risk factor, both in ckd and the general population. Several reports have been showing that oxidative status is consistently elevated in CKD patients [3, 6, 7, 28-31]. Plasma levels of oxidative stress biomarkers, such as thiol levels, lipid peroxidation products, oxidized proteins levels, modified aminoacid residues, all point to a systemic oxidative stress. As a consequence, vasculature becomes an important target to uremic environment and toxins, suffering oxidation and inflammation and accounting for the high mortality rate due to CVD reported for CKD population. Vascular injury, such as endothelial dysfunction and atherosclerosis, is well documented in CKD patients [32-35]. We recently demonstrated that CKD vessels are markedly calcified and nitrated [36], in agreement with the vascular calcification [37] and nitration [38] observed in experimental models. However, CKD is a complex disease, associated with several co morbidities/pathologies, such as a chronic inflammatory state, hypertension, etc [33, 39-41]. Thus, elucidating the mechanisms underlying the vascular physiopathology of CKD is not a straightforward task. Here, we aimed to investigate the redox effects of uremia *per* in EC. For this purpose, isolated cells in culture seem to be a suitable model to answer such question. Using this model, uremic toxins have been shown to trigger several cellular responses. Small toxins (500-2000 Da) are able to inhibit hepatoma cell line to synthesize and secrete apolipoprotein A [42]. Uremia induces vascular calcification [35, 43, 44], likely by up-regulating osteopontin [13] and osteocalcin expression [14] in addition to alkaline phosphatase activity [14] in smooth muscle cells in culture. EC are also sensitive to uremic toxins. Authors have been demonstrating that uremic serum induces the expression of adhesion molecules [16], increases proliferation and alters morphology [15] and hemostatic properties of EC [45, 46]. Our data showed that uremic serum significantly ROS production by EC early after 3h, indicating that an oxidative metabolism has taken place in the very beginning of the exposure. Several cellular sources of ROS have been described. We have focused the NADPH oxidase as a likely important source of ROS, however, in vivo other ROS sources cannot be excluded, such as mitochondrial respiratory chain and other oxidases [23, 47]. The redox activity of uremic toxins, particularly indoxyl sulfate, has been recently addressed. Indoxyl sulfate induces ROS production in both mesangial cells [48] and HUVEC [49], as measured by 2',7'-

dichlorodihydrofluorescein (DCFH). In the latter work, detected ROS was shown to be derived from NADPH oxidase activation [49]. While DCFH mostly reacts with hydrogen peroxide, DHE preferentially reacts with superoxide, and both of which are products of this enzymatic complex.

In addition to ROS production, RNS were also investigated. Three hours treatment with the uremic serum did not affect NO production. After 24h, nitric oxide bioavailability was increased in EC exposed to non-uremic serum, but remained unchanged in those cells exposed to uremic serum. This result indicates that uremic environment promotes a decreased NO bioavailability, which might be relevant to the accelerated atherogenesis in CKD [50]. In this sense, NOS inhibition by ADMA and/or eNOS uncoupling induced by uremia [51] could be responsible for the decreased NO synthesis. However, the findings that iNOS expression was not changed by uremia and that higher levels of protein nitration were measured in uremic conditions suggest that uremic toxins interfered in the normal course of NO availability, by increasing its consumption rate [52. It is well recognized that NO reaction with superoxide produces nitrating species, such as peroxynitrite {Radi, 2004 #92]. In this context, data shown here suggest increased production of ROS and RNS by uremia-stimulated EC, promoting a nitroxidative stress 24h after the initial exposure.

In addition, since literature has been considering that perturbations in cellular redox homeostasis may be an upstream event to promote ER stress [53-55], it is possible that the ER stress observed at 24h-treatment is a consequence of increased ROS and RNS formation. Indeed, ER stress has already been associated with atherosclerotic disease [56], can be induced by peroxinitrite [57] and inhibited by nitric oxide [58].

A second interesting point disclosed here is the demonstration that NAC is an efficient antioxidant to inhibit oxidative stress induced by uremia, particularly concerning NADPH oxidase activity and GRP78 and GRP94 expression. These results confirm the beneficial effect of NAC in experimental CKD models [59] and in clinical trials [60] [61], and suggest the pathway in which NAC may interfere. NAC is an antioxidant, acting both direct and indirectly. Direct action is due to its reductive potential, scavenging oxidants, such as hydroxyl radicals and hypochlorous acid [62]. Indirect action is dependent on GSH synthesis, since NAC is a precursor of GSH, also a potent antioxidant. Here, NAC seems to be effective only in those processes dependent on superoxide but not on nitric oxide release, suggesting its ability to inhibit superoxide

formation and consequently nitrotyrosine and ER stress. Inhibition of superoxide production, however, may not due to a direct NAC scavenging, since the rate constant for this reaction is negligible under physiological conditions [62]; rather, a thiol-based NADPH oxidase inhibition should be envisioned as the molecular mechanism. Indeed, vascular NADPH oxidase seems to be regulated by its thiol status [63]. ER stress and nitrotyrosine formation inhibition by NAC pretreatment may be a consequence of either NAC or GSH reaction with peroxynitrite [64]. Recently, our group described an intense nitrotyrosine staining in CKD patients arteries, in contrast to a much lower degree found in control arteries [36]. Thus, it would be interesting to assess if NAC-treated CKD patients would present a lower vascular nitrotyrosine level than a placebo CKD population.

In summary, our findings show that uremic serum induces an increased production of superoxide and protein nitration, in addition to ER stress in EC. These events are all inhibited by a single pre-exposure to NAC. Altogether, these data suggest that superoxide formation and its further reactions may be the first oxidative mechanism triggered by uremic toxins, which may contribute to endothelial dysfunction in CKD. In addition, NAC effects shown here provide the molecular basis for the observed clinical benefits of NAC in renal diseases.

METHODS

Uremic and non-uremic sera pool

Uremic sera pool was obtained from 43 patients with end-stage renal disease on maintenance hemodialysis (22 male and 21 female). Their mean age was 49±2 years (26-79 years). Causes of renal failure were: chronic glomerulonephritis (n=16), nephrosclerosis (n=8), hypertensive nephrosclerosis (n=6), polycystic kidney disease (n=2), chronic pyelonephrosis (n=1), lupus (n=2), chronic insufficiency (n=7) and transplant rejection (n=1). Non uremic (control) sera pool was obtained from 50 subjects, gender- (faixa), age- (faixa) and dyslipidemia-paired with the uremic sera pool profile. Diabetes mellitus, malignancy, or infection diseased individuals were not included in the study. Eight mL blood were aseptically collected from fasted individuals in gel-containing tubes. This protocol was approved by the Pontíficia Universidade Católica do Paraná Ethics Committee, and informed consent to serum utilization was signed by all participants. Uremic and non uremic sera were pooled and inactivated.

Sera pool characterization

Biochemical parameters. Lipid profile, ions, glucose, urea, uric acid, creatinine, albumin and C-reactive protein levels were determined with commercial kits in a clinical laboratory.

Oxidative stress biomarkers. Total thiols and carbonylated proteins were assayed by Ellman's reagent [65] and DNPH method [66], respectively.

Rabbit aortic endothelial cell culture

Rabbit aortic endothelial cells (EC) were routinely cultivated in F12 medium containing 10% fetal bovine serum (FBS) and 40μg/ml gentamycin, at 37ºC in a 5% CO_2 humidified incubator. Cell detachment was obtained with pancreatin [67]. To the assays, 0.5% SFB-starved EC were incubated with growth media containing 10% uremic or non-uremic pools for 3 and 24 hours, except when otherwise indicated. When stated, EC were exposed to 2 mM N-acetylcysteine (NAC) during 12 hours before starvation and treatment.

NAD(P)H oxidase activity in EC membrane fractions

NADPH oxidase activity was assessed by DHE-derived fluorescence in a microplate reader, as described [Fernandes]. EC were grown in 100 mm dishes and treated with 10% uremic or non uremic sera pools. After 3 hours, cells were washed with cold PBS, homogenized in lysis buffer (50 mM Tris pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride), sonicated and centrifuged (18,000×*g* / 15 minutes). Supernatants were ultracentrifuged (100,000 × *g* / 1 hour) to isolate the membrane-enriched fraction. Membrane fractions (10 μg of protein) were incubated with 10 μM dihydroethidium (DHE), 1.25 μg/mL DNA and 50 μM NADPH in PBS. Incubations were performed for 30 minutes at 37°C. Fluorescence was read in acridine filter (λ_{ex}=490 nm and λ_{em}=570 nm) in a microplate fluorescence reader (Wallac Victor2 1420-Multilabel Counter, USA, PerkinElmer ™). In some experiments, 25 U/mL SOD or 2 μM diphenyleneiodonium (DPI) was included in reaction mixture.

Nitric oxide availability analysis by DAF oxidation

Nitric oxide production was estimated by DAF-derived fluorescence [ref]. EC were grown in 60 mm dishes and treated with 10% sera pools, during 3 and 24 hours. In the last 30 min, 5 μM diaminofluorescein diacetate (DAF) was added. Cells were detached, washed and resuspended in PBS. Flow cytometry data were collected on a FACScan (BD, San Jose, CA). The mean fluorescence (FL) of at least 10,000 events was measured at a low flow rate. Analysis was carried out using Cell Quest software (Becton Dickinson, Rutherford, NJ, USA). NO production by EC has been reported as stimulation index, which is the ratio of the mean fluorescence of the stimulated and unstimulated cells [68].

Nitrotyrosine levels analysis by dot blot

EC treated with uremic and non-uremic sera pools for 3 and 24 hours were lysed in a lyse buffer. Five μg of supernatant proteins were blotted onto a PVDF membrane (Millipore). A polyclonal anti-nitrotyrosine (1:500, Sigma) and anti-rabbit IgG conjugated with HPR (1:500, Sigma) were used. Blots were developed with a quimioluminescent kit West Pico (Pierce). Results were analyzed by dot densitometry in

an image program (ImageJ). The same procedure was performed to uremic and non-uremic sera pools.

Western Blot analyses

EC were incubated with sera pools 10% for 3 and 24 hours, with or without NAC pre-treatment, to detect changes in expression of inducible NO synthase (iNOS), KDEL endoplasmic reticulum proteins, and β-actin. Whole-cell lysates were prepared, mixed with a sample buffer (100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue) and subjected to a 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked and incubated with monoclonal anti-iNOS (1:800, Sigma), monoclonal anti-KDEL (1:800, Stressgen), and monoclonal anti-β-actina (1:4000, Sigma) antibodies according to the manufacture's recommendations. HRP-conjugated secondary antibodies were from KPL (1:1000). Reactions were developed with a quimioluminescent kit WestPico (Pierce). Results were analyzed by bands densitometry and values were normalized by β-actin band densitometry.

Statistical analysis

Results are expressed as mean ± SEM. Statistical differences were analyzed using Student's t test for paired data (Origin). A $p<0.05$ was considered statistically different.

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Table 1: Sera pools characterization

	Non-uremic	Uremic
Sodium (mmol/L)	138	137
Potassium (mmol/L)	4.4	6.9
Urea (mg/dL)	30	165
Glucose (mg/dL)	82	80
Calcium (mg/dL)	8.4	8.3
Phosphorus (mg/dL)	4.0	6.7
Total Cholesterol (mg/dL)	178	135
Triglyceride (mg/dL)	188	125
Albumin (g/dL)	4.1	3.6
Uric acid (mg/dL)	4.8	7.4
HDL (mg/dL)	48	41
LDL (mg/dL)	92	69
Creatinine (mg/dL)	0.9	10
"C" reative protein (mg/dL)	0.69	1.3
Total thiols (μM)	1620	580
Protein carbonyl (nmol/mg albumin)	0.0134	0.0192
Nitrotyrosine (au/μg protein)	20	26

Figure 1. DHE oxidation by EC membrane fractions. EC were incubated with 10% non-uremic (□) or uremic(■) sera pool for 3 hours. In some experiments, EC were pre incubated with 2 mM NAC. Membrane fractions were incubated with 50 µM NADPH, 10 µM DHE, 1.25 µg/mL DNA in PBS during 30 min at 37°C, in the presence or absence of 25U/mL SOD or 2 µM DPI. Fluorescence (λ_{em}=490 nm, λ_{ex}=570 nm) was read in a microplate reader. * P<0.05

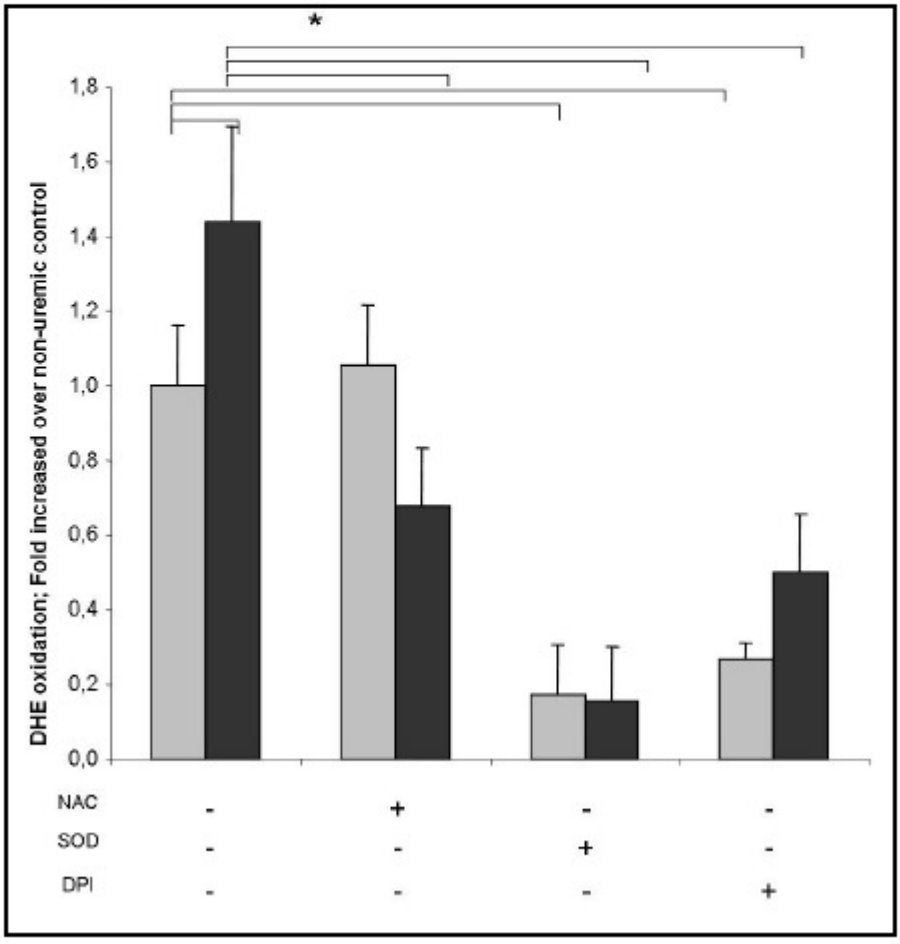


Figure 2: NO bioavailability in EC exposed to uremic and non uremic sera pools. EC were incubated with 10% non-uremic (□) or uremic(■) sera pool for 3 hours. In some experiments, EC were pre incubated with 2 mM NAC. In the last 30 min, 5 μM DAF were added. Cells were detached, washed and ressuspended in PBS. Flow cytometry data were collected. * $p<0.05$.

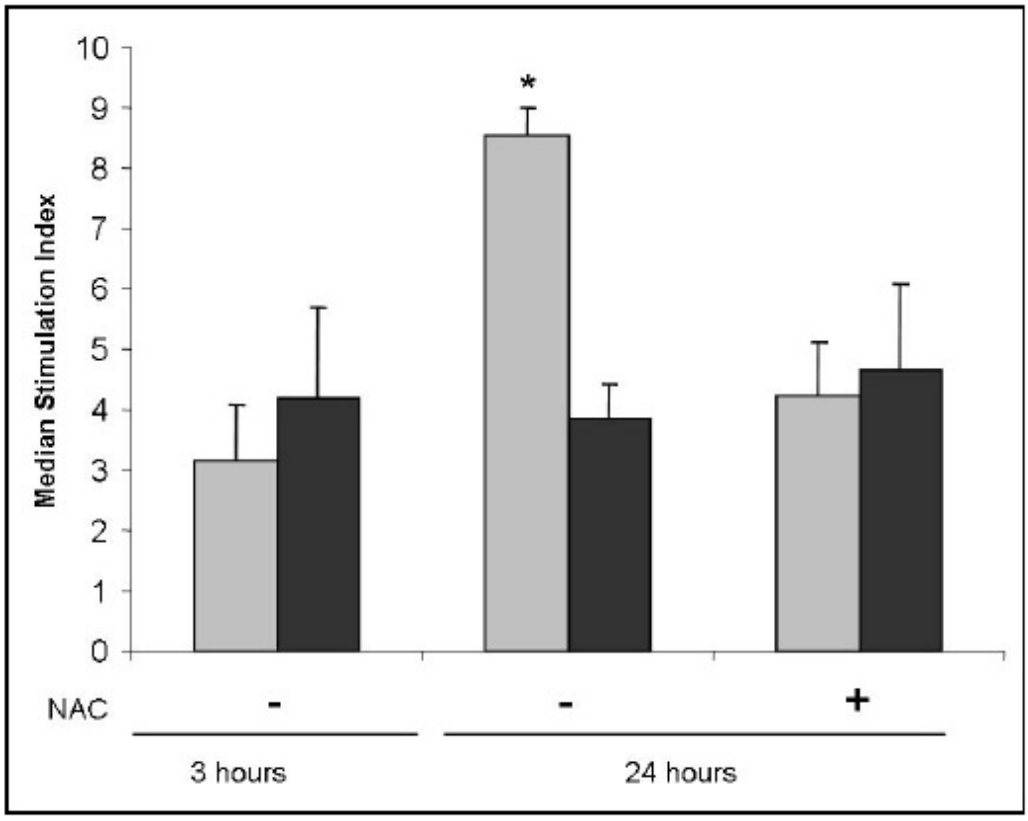
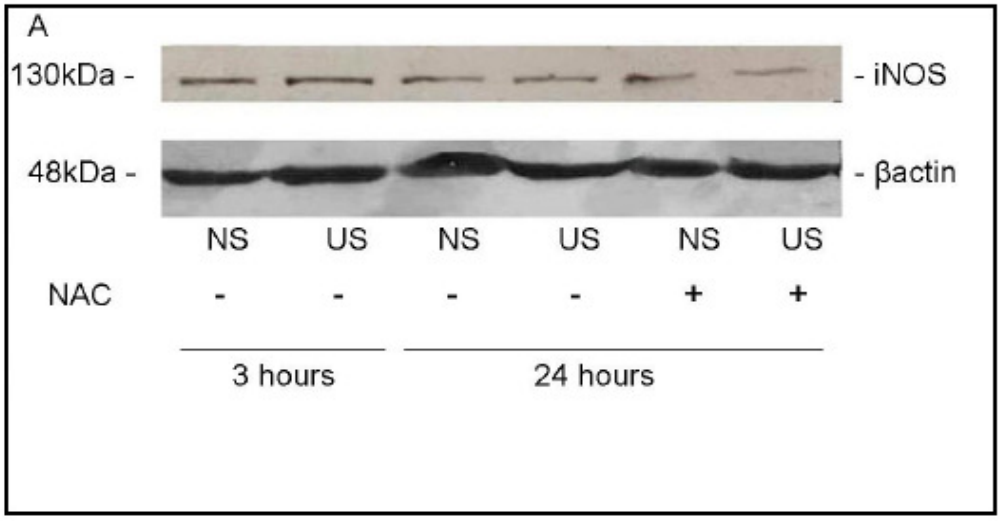


Figure 3. iNOS expression by EC exposed to uremic and non uremic sera pool..

Protein lysates of EC (pre-treated or not with NAC) incubated with non-uremic (NS, □) or uremic (US, ■) sera pool during 3 or 24 h were electrophoresed in a 10% SDS-PAGE. Western blot was performed with anti-iNOS and β-actin antibodies. A representative western blot is shown (A). Results are shown as iNOS and β-actin bands densities ratio (B).



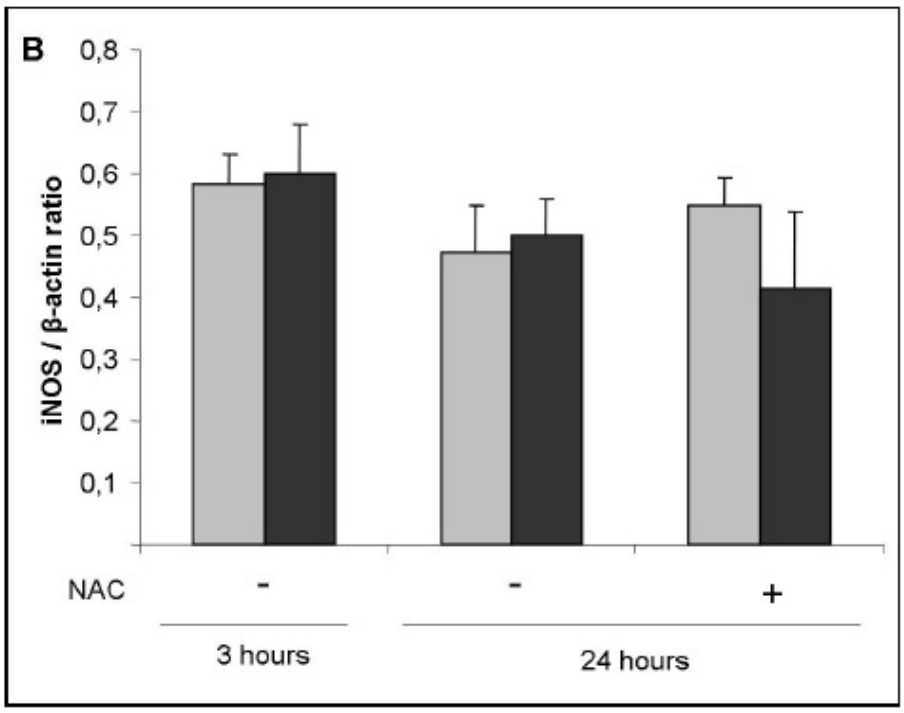
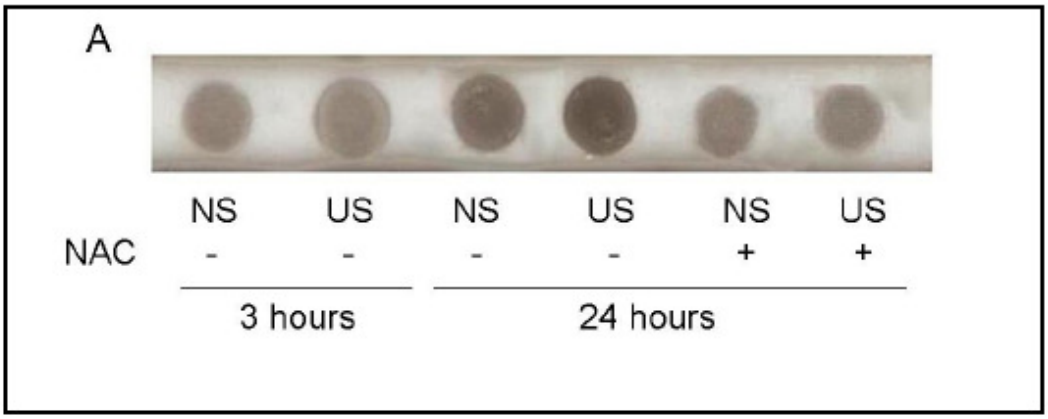


Figure 4. Nitrotyrosine residues in EC exposed to uremic and non uremic sera pool. 5 µg protein lysates of EC (pre-treated or not with NAC) incubated with non-uremic (NS, □) or uremic (US, ■) sera pool during 3 or 24 h were blotted onto a PVDF membrane and reacted with anti-3-nitrotyrosine antibody. A representative assay is shown (A). Results are shown as dot densities (B). *P<0.01 ; ** P<0.05.



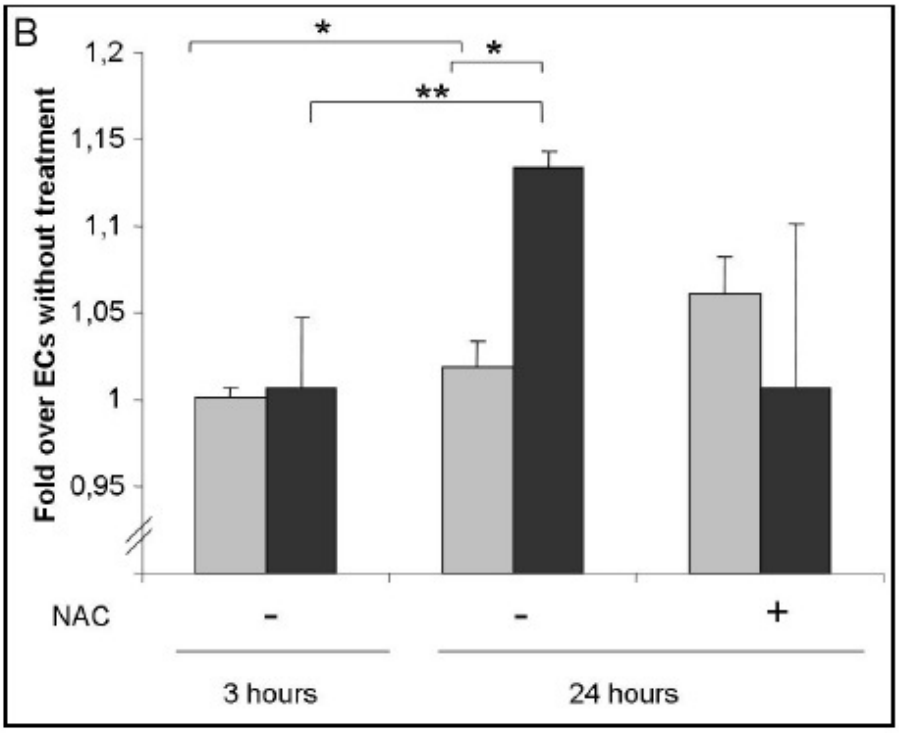
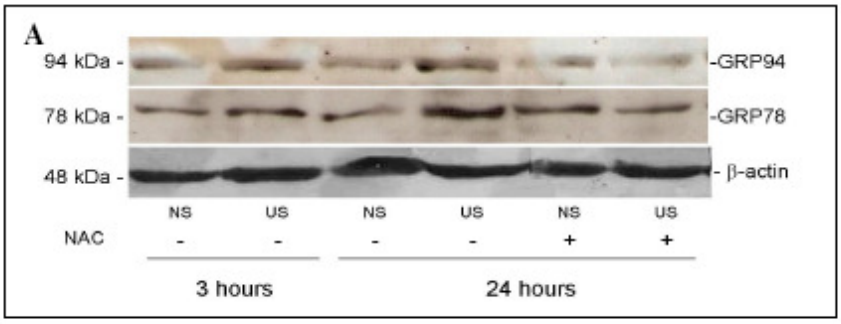
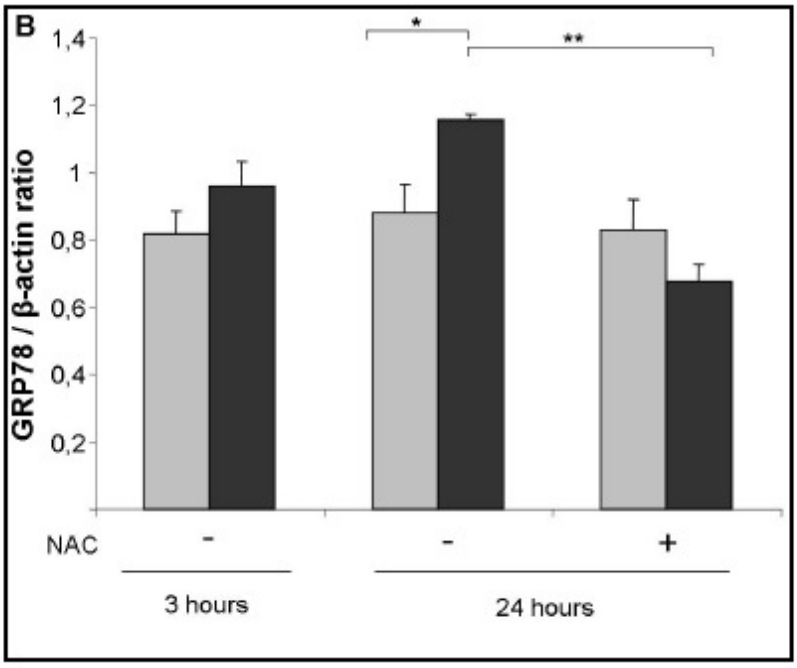
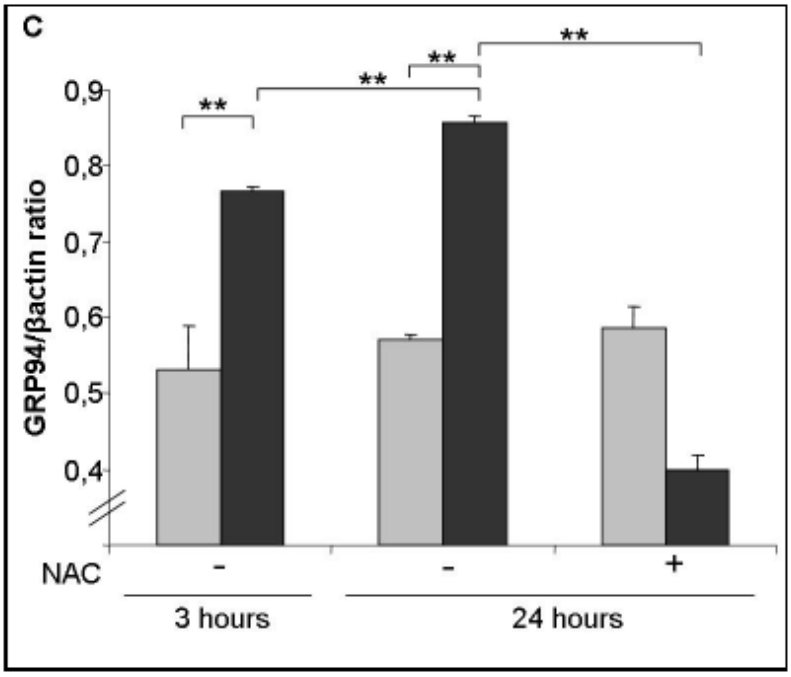


Figure 5. GRP78 and GRP94 chaperones expression by EC exposed to uremic and non uremic sera pool. Protein lysates of EC (pre-treated or not with NAC) incubated with non-uremic (NS, □) or uremic (US, ■) sera pool during 3 or 24 hours were electrophoresed in a 10% SDS-PAGE. Western blot was performed with anti-KDEL and β-actin antibodies. A representative western blot is shown (A). Results are shown as GRP78 and β-actin bands densities ratio (B). Results are shown as GRP94 and β-actin bands densities ratio (C). * $p<0.05$; **$P<0.01$.






C
**
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0,9
0,8
0,7
0,6
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0,4
GRP94/βactin ratio
NAC
-
-
+
3 hours
24 hours

5. CONCLUSÃO

A DCV é uma conseqüência importante da DRC [7, 73]. Diversos estudos têm mostrado que eventos cardiovasculares, entre eles a doença aterosclerótica arterial coronariana (DAC) e a hipertrofia ventricular esquerda (LVH), são responsáveis pela mortalidade prematura em mais de 50% dos pacientes ESRD [7, 73]. Além disso, o risco de morte em pacientes de diálise é sempre maior do que na população geral, chegando a ser centenas de vezes maior em pacientes portadores de DRC de aproximadamente 30 anos de idade comparado a adultos sem a DRC e na mesma faixa etária [74]. Estes dados indicam que os fatores de risco tradicionais (chamado fatores de risco de Framingham) para DCV, não são os únicos determinantes quando se trata da população renal crônica. De fato, vários fatores de risco não tradicionais associados a DRC têm sido considerados para explicar a extensão e a gravidade das complicações cardiovasculares nestes pacientes. Dentre esses, inflamação, desnutrição, hiperhomocisteinemia, anemia, distúrbios no metabolismo de cálcio de fósforo e estresse oxidativo parecem ter impacto sobre a aceleração de processos arterosclerótico, por mecanismos moleculares ainda desconhecidos. Assim, entender a fisiopatologia de DCV na DRC é primordial para se tentar inibir a mortalidade associada a eventos cardiovasculares nos pacientes com ESRD.

No presente trabalho, dois destes fatores de risco não tradicionais foram primeiramente estudados: a calcificação vascular e a nitração de proteínas vasculares. A calcificação não está presente tradicionalmente em doença vascular na população em geral, apenas em pacientes idosos e diabéticos [75]. Em nosso estudo foi observada a calcificação vascular acentuada em pacientes DRC jovens, assintomáticos e com poucos fatores de risco tradicionais de DCV. A marcação de nitrotirosina nesses pacientes também se apresentou elevada, correlacionado positivamente com a calcificação. Estes dados mostram que tanto a calcificação vascular como estresse oxidativo nos pacientes renais crônicos são eventos precoces da lesão aterosclerótica, merecendo estudos mais detalhados.

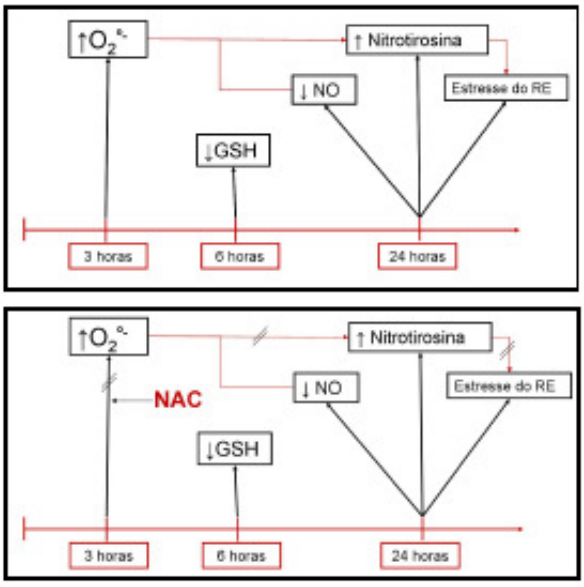
Como uma das características da DRC é o acúmulo de toxinas urêmicas no sangue, e como é reconhecido que vários componentes das toxinas urêmicas disparam diversas respostas celulares [17, 20, 21, 76, 77], decidimos investigar o papel da uremia *per se* na indução de estresse oxidativo em células endoteliais. Nossos estudos mostraram que a uremia ativa a formação de espécies reativas pelas células endoteliais.

Identificamos a participação do complexo NADPH oxidase como uma fonte de radicais superóxido após 3 horas de exposição às toxinas urêmicas, juntamente com um aumento dos níveis de GSH intracelular, quando comparados ao controle. Este aumento talvez seja uma resposta primária à produção de espécies reativas, possivelmente induzida pela via Nrf2/Keap-1. Após 6 horas já há a diminuição da concentração de GSH intracelular, mostrando que nesse tempo pode estar se instalando o estresse oxidativo. E em 24 horas outras formas de estresse se mostram detectáveis, como o estresse do retículo endoplasmático e o estresse nitroxidativo. Verificamos também que, neste modelo, a NAC mostrou-se um antioxidante eficaz, dando suporte aos achados clínicos [59].

Outras fontes de estresse oxidativo, bem como a melhor elucidação de como a uremia altera a biodisponibilidade do NO, são pontos a serem estudados futuramente.

Juntos estes dados sugerem que a formação de superóxido e suas reações conseqüentes podem ser o primeiro mecanismo engatilhado pelas toxinas urêmicas, tendo o endotélio como alvo importante, contribuindo para aterogênese precoce nos pacientes DRC, conforme proposto na figura 3.

Figura 3: Esquema proposto para a atuação do superóxido no endotélio vascular, na presença e ausência de NAC.



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7. ANEXOS

7.1 ESTUDOS COMPLEMENTARES

7.1.1. Viabilidade e Proliferação.

Para verificarmos possíveis efeitos tóxicos dos soros humanos não urêmico e urêmico nas células endoteliais de coelho, 3 x 10^4 células foram plaqueadas em placas de 24 poços. Após adesão, foram incubadas com meio F12 contendo 10% dos pools de soros ou 10% soro fetal bovino (SFB) e antibióticos. Após 24 e 48 horas as células foram soltas com tripsina e 50µL da suspensão foi misturada a 50µL de azul de tripan. Células viáveis e não viáveis foram contadas em câmara de Neubauer em microscópio.

A exposição de células endoteliais a meio contendo pools de soros humanos não causou alterações na taxa de proliferação (Figura 1A), nem na taxa de viabilidade celular quando comparadas ao soro fetal bovino (Figura 1B).

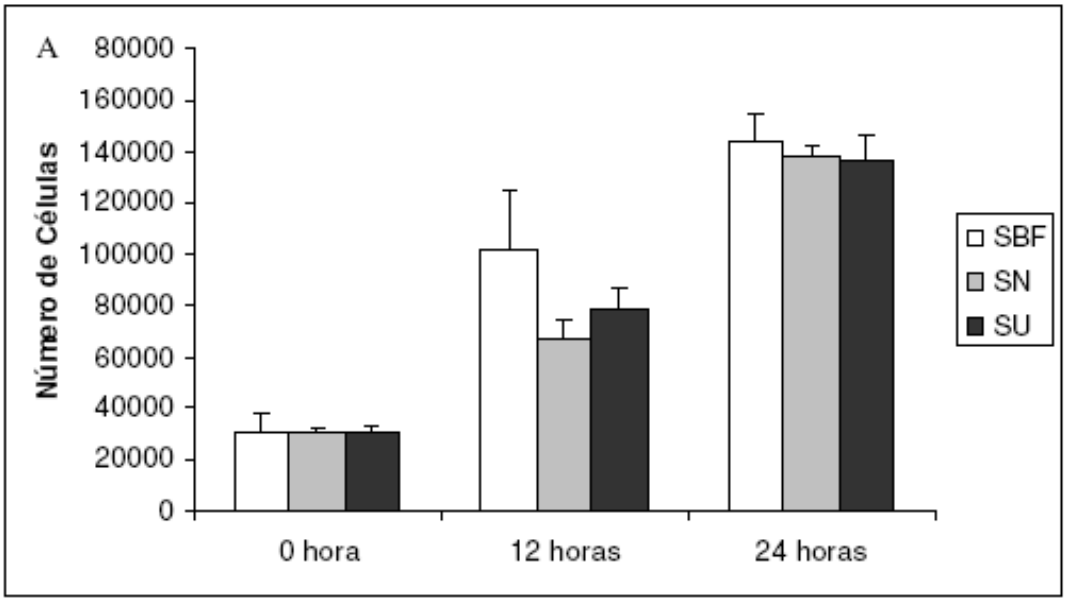
Esses dados contrastam com os dados de Serradell [17], os quais mostraram que o meio urêmico causa um aumento na velocidade de proliferação celular de cordão umbilical. Entretanto, esses autores usaram uma concentração de 20% de soro e um tempo de exposição de 48-72h. Como a intenção deste ensaio foi mostrar que o soro humano não era tóxico para células de coelho, nossos experimentos prosseguiram.

7.1.2.Análise do GSH intracelular por DTNB

As células foram plaqueadas em placas de 100 mm e após a adesão foram tratadas com meio F12 contendo 10% dos pools de soro urêmico e não urêmicos. Após 3 e 6 horas, as células foram coletadas e centrifugada a 4◦C. Em seguida, o pellet foi ressuspendido e homogeneizado em solução de lise gelado (ácido tricloroacético 5% e DTPA 0,5 mM), centrifugado e o sobrenadante separado para a análise. O GSH foi então determinado espectrofotometricamente (412 nm), após reação com DTNB [78]. A quantificação do produto TNB foi feita com uma curva de calibração obtida com GSH autêntico. Padronizou-se a quantidade de GSH em µmol por µg de proteína.

A exposição de RAECs ao soro urêmico causou intensa depleção nos níveis de GSH (0,77 ± 0,11 µmol GSH/µg proteína) após 6 horas, quando comparadas às expostas ao pool de soro não-urêmico (1,19 ± 0,02 µmol GSH/µg proteína, p=0,02), indicando um estresse oxidativo induzido pela uremia nestas células (Figura 2). Em 3 horas, o conteúdo de GSH intracelular se mostrou aumentado nas células tratadas com soro urêmico (1,33 ± 0,24 µmol GSH/µg proteína, vs soro não-urêmico em 3 horas 0,97 ± 0,19 µmol GSH/µg proteína, p=2,77)

Figura 4: Proliferação de células endoteliais de coelho induzida pelo pool controle não urêmico (SN; ▒), pool urêmico (SU; ■) e soro fetal bovino (SFB; □). Células foram plaqueadas em placas de 24 poços (3×10^4 célula / poço.) e cultivadas com meio contendo 10% dos pool de soros (urêmico e não urêmico) ou SFB. Após o tempo de incubação as células foram contadas. Os resultados foram expressos em média ± desvio padrão da média. Não houve diferenças significativas na proliferação das células endoteliais, sob os tratamentos, nos devidos tempos. A – Proliferação, em numero de células; B – Viabilidade em %.



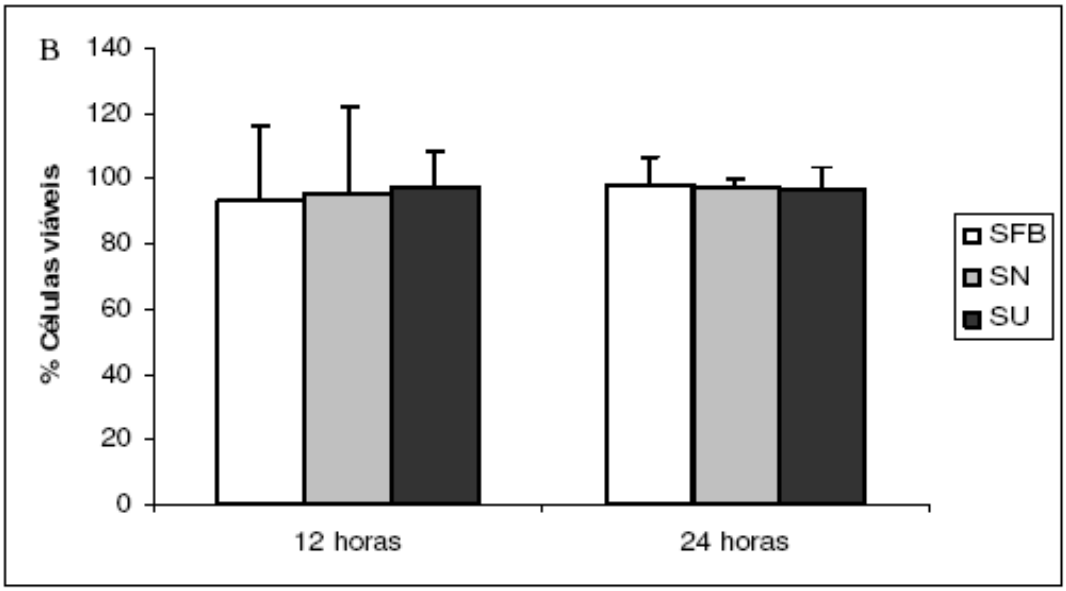
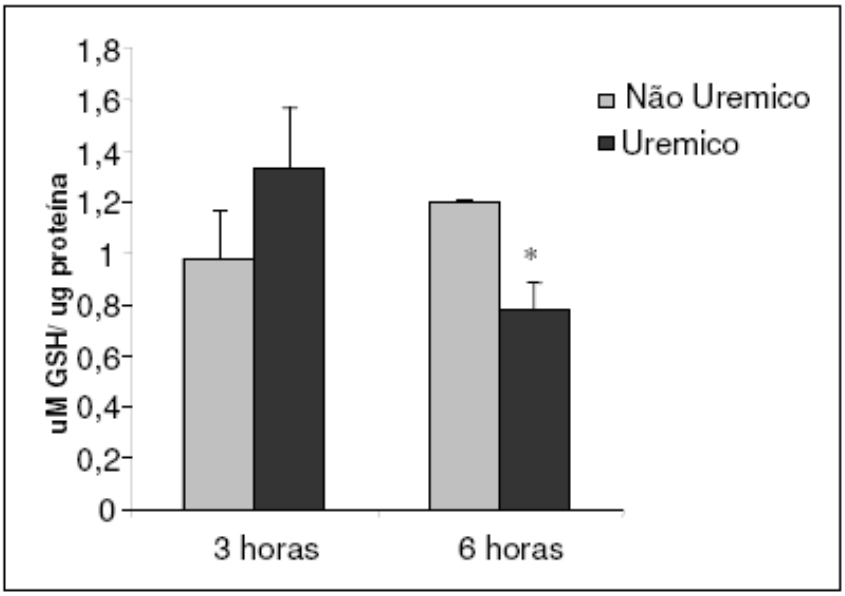


Figura 5: Determinação da concentração de GSH em células endoteliais de aorta de coelho (RAEC). As células foram plaqueadas em placas de 100 mm e tratadas, durante 3 e 6 horas, com 10% de pools de soro urêmico e 10% de pool de soro não urêmico. A concentração de GSH foi determinada espectrofotometricamente (412 nm) após reação com DTNB. *P=0,02 vs. ECs tratadas com pool não urêmico por 6 horas.



7.2 APROVAÇÃO DO COMITE DE ÉTICA EM PESQUISA

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa e Pós-Graduação

Curitiba, 26 de junho de 2005
Of. 226/05/CEP-PUCPR

Ref. "Modulação fenotípica induzida por soro urêmico em células vasculares".

Prezado (a) Pesquisador (es),

Venho por meio deste informar a Vossa Senhoria que o Comitê de Ética em Pesquisa da PUCPR, no dia 01 de junho do corrente ano aprovou o Projeto Intitulado "Modulação fenotípica induzida por soro urêmico em células vasculares", pertencente ao Grupo III, sob o registro no CEP nº 577 e será encaminhado a CONEP para o devido cadastro. Lembro ao senhor (a) pesquisador (a) que é obrigatório encaminhar relatório anual parcial e relatório final a este CEP.

Atenciosamente,

Profª M. Sc Ana Cristina Miguez Ribeiro
Coordenadora do Comitê de Ética em Pesquisa - PUCPR

Ilma Sra.
Lia Sumie Nakao

Rua Imaculada Conceição, 1155 - Prado Velho - CEP 80215-901 - Caixa Postal 16210
http://www.pucpr.br - Telefone: (41) 3271-1543 - Telefax: (41) 3332-6886 - Curitiba - Paraná - Brasil

7.3 TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Eu, , portador de RG # , manifesto aqui meu consentimento em participar da pesquisa intitulada **"Modulação fenotípica induzida por soro urêmico em células vasculares"**, sob responsabilidade da Dra. Lia S. Nakao, da Pós-Graduação em Ciências da Saúde, Centro de Ciências Biológicas e da Saúde, PUCPR.

Estou ciente de que:

(i) tal pesquisa poderá abrir novas perspectivas quanto ao entendimento da evolução de doenças vasculares associadas à doença renal crônica, e que a utilização de meu sangue poderá beneficiar outras pessoas a um médio-longo prazo;

(ii) o sangue será coletado de uma veia de meu antebraço por profissional habilitado e a quantidade coletada (5-10ml) não causará repercussão sobre meu estado de saúde, gerando apenas o desconforto na hora da coleta.

(iii) poderei a qualquer momento (desde o início até o final da pesquisa, ie, agora até a publicação do artigo científico) solicitar esclarecimentos sobre a pesquisa;

(iv) tenho a liberdade de recusar a participar ou retirar meu consentimento, em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo;

(v) minha privacidade está garantida quanto aos dados confidenciais envolvidos na pesquisa;

(vi) estou doando, e não vendendo, meu sangue, e portanto, não receberei dinheiro por tal ato.

Estando de acordo com tal termo, firmo aqui,

Nome, data e assinatura.
