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DESENVOLVIMENTO FETAL E FUNÇÃO RENAL EM RATOS SUBMETIDOS A UMA SOBRECARGA DE SÓDIO DURANTE A VIDA PRÉ-NATAL

HENRIQUETA DIAS CARDOSO

Recife-PE
Fevereiro-2009

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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.
Orientadora: Prof.ª Dr.ª Ana Durce Oliveira da Paixão

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"Desenvolvimento fetal e função renal em ratos submetidos a uma sobrecarga de sódio durante a vida pré-natal"

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DEDICATÓRIA

A minha família, com carinho aos meus irmãos, *in memoria* a Moisés Dias Cardoso e Oséas Dias Cardoso.

Aos meus queridos pais, Maria José Dias Cardoso e Rosalvo Cardoso.

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“A tarefa não é contemplar o que ninguém contemplou, mas meditar, como ninguém ainda meditou, sobre o que todo mundo tem diante dos olhos.”

Shopenhauer

LISTA DE ABREVIATURAS E SIGLAS

AT1- Receptor para a angiotensina II tipo 1

AT2- Receptor para a angiotensina II tipo 2

C- Control group

EPM – Erro padrão médio

FF – Fração de filtração

FG – Filtração glomerular

FPR – Fluxo plasmático renal

FSR – Fluxo sangüíneo renal

g- grama

GFR - Glomerular filtration rate

h - Horas

Hct- hematocrit

i.p- Intraperitonial

IGF- Fator de crescimento dependente de insulina

K^+ - Íon potássio

KCl- Cloreto de potássio

M- mol

MDA – Malonildialdeído

min - Minuto

mM- mili mol

Na^+ - Íon sódio

NN – Número de néfrons

nº - Número

Ox- Estresse oxidativo

Pág. - Página

PAM – Pressão arterial média

PC – Peso corpóreo

PR – Peso renal

RBF - Renal blood flow

RNA – Ácido ribonucléico

ROS- Substâncias reativas de oxigênio

RVR – Resistência vascular renal

SP- Saline group

SPL- saline during the prenatal and lactation (L)

SRAA – Sistema renina – angiotensina – aldosterona

TBARS - Substâncias reativas ao ácido tiobarbitúrico

TNF-α- Fator de necrose tumoral- alfa

UFPE – Universidade Federal de Pernambuco

$U_{Prot24h}$ - 24h-urinary protein

V – Fluxo urinário mensurado durante hemodinâmica renal

SUMÁRIO

RESUMO

Evidências apontam uma relação inversa entre o crescimento pré-natal e doença renal no adulto. Neste estudo foram avaliados parâmetros correlacionados com o desenvolvimento fetal, tais como estresse oxidativo (Ox) placentário e volume plasmático (VP) de mães mantidas com sobrecarga de sódio. Adicionalmente, a função renal da prole destas mães foi avaliada na idade adulta. Ratas Wistar foram mantidas com uma solução de cloreto de sódio 0,17 M, em substituição a água de beber, a partir de vinte dias antes do acasalamento até o vigésimo dia de prenhez/parto ou até o final da lactação. O Ox de tecidos maternos e da prole, o VP e a proteinúria 24h (Uprot-24h) foram medidos através de métodos colorimétricos. A prole, ratos machos, aos 90 dias de idade teve a hemodinâmica renal avaliada através de transdutor de pressão sangüínea, sonda de fluxo e clearance de inulina para mensurar a pressão arterial média (PAM), fluxo sanguíneo renal e ritmo de filtração glomerular respectivamente. O número de néfrons (NN) foi contado em suspensão renal. As mães não apresentaram alterações de VP, Ox placentário, bem como peso fetal, contudo, Uprot 24h esteve (150%, $p<0,05$) mais elevada. O grupo submetido à sobrecarga de sódio durante o período pré-natal apresentou Uprot-24h aumentada (45%, $p<0,05$) e PAM, hemodinâmica renal, NN, Ox renal inalterados. O grupo submetido à sobrecarga de sódio adicionalmente durante a lactação mostrou aumento de Uprot-24 h (27%, $p<0,05$), Ox renal (44%, $p<0,05$), redução do RFG (12%, $p<0,05$), aumento do VP (26%, $p<0,05$) e PAM, NN inalterados. Embora que entre os parâmetros maternos avaliados apenas a proteinúria tenha se apresentado elevada e o crescimento fetal não tenha apresentado retardo, os animais submetidos à sobrecarga de sódio durante o período pré-natal apresentaram alteração da função renal na idade adulta. Portanto, a sobrecarga de sódio expandida durante a lactação exacerbou as alterações produzidas durante o período pré-natal.

Palavras chaves: desenvolvimento fetal, sobrecarga de sódio, fluxo sangüíneo renal, ritmo de filtração glomerular, placenta, estresse oxidativo.

ABSTRACT

Evidence points to an inverse relationship between prenatal growth and adult renal disease. This study evaluated factors correlated with fetal development, such as placental oxidative stress (Ox) and plasma volume (PV) of dams on sodium overload. Moreover, it evaluated the renal function of adult offspring from these dams. Wistar dams were maintained on a sodium chloride solution 0.17 M, instead drinking water, from twenty days before mating until either twentieth pregnancy day/parturition or weaning. Maternal and offspring tissue Ox, PV and 24h-urinary protein ($U_{Prot24h}$) were measured using colorimetric methods. The male offspring at age of 90 days had its renal hemodynamics evaluated using a blood pressure transducer, a flow probe and inulin clearance to measure respectively, mean arterial pressure (MAP), renal blood flow and glomerular filtration rate (GFR). The number of nephrons (NN) was counted in kidney suspension. Dams showed unchanged PV and placental Ox, as well as unchanged fetal weight, but increased $U_{Prot24h}$ (150%, $p<0.05$). Prenatally sodium overloaded rats presented increased $U_{Prot24h}$ (45%, $p<0.05$) and unchanged MAP, renal hemodynamics, NN and kidney Ox. Prenatally and postnatally sodium overloaded rats showed increased $U_{Prot24h}$ (27%, $p<0.05$) and kidney Ox (44%, $p<0.05$), reduced GFR (12%, $p<0.05$), increased PV (26%, $p<0.05$) and unchanged MAP and NN. Although just maternal proteinuria was changed and fetal growth retardation was not seen, prenatally sodium overloaded rats showed renal function alteration at adult life. Salt overload from prenatal to weaning leads to further renal function alterations than those seen in prenatal overloaded rats.

Keywords: fetal development . salt overload . renal blood flow . glomerular filtration rate . placenta . oxidative stress.

1-INTRODUÇÃO

Programação pré-natal

O crescimento intra-uterino é controlado por uma complexa inter-relação de fatores maternos e fetais, incluindo os sistemas circulatório, endócrino e a função metabólica (BYRNE e*t al*., 2000). O desenvolvimento do concepto apresenta determinados períodos de rápida divisão celular. Eventos externos durante esta fase podem alterar a estrutura e a função de órgãos e tecidos em momento precoce do desenvolvimento do feto (BARKER, 1994). Assim, perturbações no ambiente fetal podem alterar a programação intra-uterina e levar a ocorrência de doenças na vida adulta (BYRNE e*t al*., 2000). A natureza e a duração dos fatores externos, sobre o feto, são determinantes para a ocorrência de respostas fisiológicas especificas, as quais podem resultar em processos patológicos (BERTRAM, C.E. & HANSON, 2001).

Estudos epidemiológicos evidenciam que o ambiente intra-uterino inadequado pode programar alterações metabólicas e predispor ao desenvolvimento de doenças crônicas na idade adulta (ERIKSSON e*t al*., 2002; BARKER e*t al*., 2006; HALLAN e*t al*., 2008). Assim, tem sido demonstrado uma conexão entre o crescimento fetal reduzido e diabetes do tipo II (HALES e*t al*., 1991), hipertensão (MANNING J & VEHASKARI, 2001) e doenças renais (HOY *et al*., 1999). Redução do número de células pancreáticas β (GAROFANO e*t al*., 1997), como também anormalidades na secreção e ação da insulina estão correlacionadas com comprometimento do desenvolvimento fetal (OSMOND & BARKER, 2000). Além destas alterações metabólicas, podem ocorrer também alterações da reatividade vascular (MARTIN e*t al*., 2000) e redução do número de néfrons (LANGLEY-EVANS e*t al*., 1999; MERLET-BENICHOU e*t al*., 1994), fatores que podem convergir para hipertensão. A oligonefrenia está

relacionada com a diminuição da área de filtração glomerular, incapacidade em excretar sódio e água, hipertrofia compensatória e alterações na hemodinâmica (PAIXÃO *et al*., 2001).

Programação pós-natal

O maior foco dos estudos epidemiológicos sobre a programação tem sido relacionado com o desenvolvimento intra-uterino. Entretanto, estudos em animais têm mostrado que o desenvolvimento pós-natal pode ter igual importância na determinação das alterações apresentadas no ser adulto (MANNING *et al.,* 2005). Alguns sistemas podem ser alterados mesmo após o nascimento como o simpático (YOUNG *et al.,* 2000), o endócrino (MEANEY *et al.,* 1996), o metabolismo lipídico (LUCAS *et al.,* 1996) e a osmoregulação (WANG *et al.,* 2003). Por outro lado, outros trabalhos apontam que somente a exposição a certos estímulos durante a segunda metade da gestação é suficiente para alterar a programação do feto (WOODS *et al.,* 2004), ou seja, algumas janelas de programação são fechadas abruptamente ao nascimento.

A maturação funcional dos rins, a qual é completada em torno do 10º dia após o nascimento (NIGAM *et al.,* 1996; REEVES *et al*., 1978), pode sofrer influência de eventos pós-natais. Considerando que há transferência de sódio através do leite materno (VIJANDE *et al*., 1996), manipulações nutricionais podem afetar o processo de maturação a ponto de modular a programação pré-natal do SRAA (MANNING *et al.,* 2005).

Manipulação do teor de sódio na dieta materna

Modificações na concentração de sódio têm sido realizadas em modelos experimentais de má-nutrição, tanto através da elevação do teor deste micronutriente (NICOLANTONIO *et*

al., 1990; CONTRERAS *et al*., 2000) quanto da redução do mesmo na dieta materna (ROY-CLAVEL *et al*., 1999). A diminuição de sódio na dieta materna aumenta, de forma anômala, a atividade SRAA (HAGEMANN e*t al*., 1994; BINDER e*t al*., 1995). A elevação da angiotensina II materna pode agir de forma direta inibindo a invasão trofoblástica (XIA e*t al*., 2002), ou indireta por promover no cotilédone aumento da produção de fator de necrose tumoral α, um potente vasoconstritor (HOLCBERG e*t al*., 2001). Estes fatores associados levam ao comprometimento da função placentária e a nutrição fetal, e assim induzir baixo peso no nascimento em prole de ratos (VIDONHO e*t al*., 2004, LOPES e*t al*., 2008). A baixa ingesta de sódio materna pode ainda desencadear disfunções no adulto como alteração do volume plasmático e elevação dos níveis séricos de triglicerídeos (ROY-CLAVEL *et al*., 1999).

Por outro lado, a suplementação de sódio na dieta, utilizada para manutenção de ratas prenhas, induz características semelhantes à pré-eclampsia como proteinúria, redução do volume plasmático e da atividade do SRAA (GARLAND *et al*., 1987; DAVEY *&* MACGILLIVRAY, 1988; BEAUSÉJOUR *et al*., 2003). A redução dos níveis de angiotensina II foi observada em placentas de ratas submetidas à sobrecarga de sódio (LEANDRO *et al*., 2008). Perturbações do SRAA podem desencadear alterações dos fatores hemodinâmicos da placenta (DAVEY *et al*.., 1988) e prejuízo do fluxo útero-placentário a ponto de reduzir o suprimento de nutrientes ao feto e assim induzir baixo peso ao nascimento (REYNODS *&* REDMER, 1995). Ademais, foi observado no 22°dia de gestação em ratas Sprague-Dawley, tratadas com suplemento de sódio, a presença de apoptose, aumento de estresse oxidativo e redução do óxido nítrico no rim materno, alterações que sugerem possíveis causas de disfunções renais maternas como a proteinúria (BEAUSÉJOUR *et al*., 2007 b).

O alto consumo de sódio, durante as fases de gestação e aleitamento, pode afetar o desenvolvimento do concepto (VIDONHO *et al*., 2004) e do neonato de forma permanente

(BALBI *et al*. 2004). Estudos realizados em animais, cujas mães receberam uma dieta rica em sal durante a gestação, revelaram que os fetos estavam expostos a altas concentrações de sódio no fluido amniótico (HAZON *et al*., 1998) e apresentavam baixo peso ao nascimento (DA SILVA *et al.,* 2003). Há evidências de que a angiotensina II encontra-se elevada nos rins de ratos adultos submetidos a uma sobrecarga de sódio durante o início da vida (DA SILVA *et al.,* 2003). Foi observado ainda que a prole adulta apresenta efeitos dismórficos mediante a elevação dos níveis de angiotensina II: os machos apresentam resistência à insulina, aumento da atividade do SRAA e dos níveis séricos de leptina (VIDONHO e*t al*., 2004; LOPES e*t al*., 2008); já as fêmeas, apresentam aumento do tecido adiposo e diminuição dos níveis séricos de leptina (LOPES e*t al*., 2008).

Peso placentário e fetal: indicadores de desenvolvimento fetal

Alterações na organogênese predispõem a prole adulta, ao desenvolvimento de hipertensão e doenças cardiovasculares (BARKER *et al*., 1996). Diversos estudos revelaram que o baixo peso ao nascimento pode influenciar no desenvolvimento de patologias como doença coronariana e distúrbios do perfil lipídico na vida adulta (BARKER , 1994). A relação entre baixo peso fetal e doença no adulto inclui facetas da síndrome metabólica representadas pela resistência à insulina (PHILIPS *et al*., 1994), disfunção vascular (MARTYN *et al*., 1995) e obesidade (YAJNIK, 2000).

Além do peso ao nascimento, o peso placentário é um indicador crucial de comprometimento da programação fetal. (CAMPBELL *et al*., 1996; MARTYN *et al*., 1995). O tamanho da placenta possui importante influência sobre a função metabólica e o sistema endócrino fetal, como a manutenção dos níveis normais de IGF-1 (OWENS *et al*., 1989) o

qual desempenha importante papel no suprimento de nutrientes destinados ao feto (GLUCKMAN & HANSON, 1995).

SRAA e gestação

Durante a gravidez normal em humanos, a última semana gestacional é caracterizada por redução da pressão sangüínea, expansão de volume e pela ativação do SRAA (DUVEKOT & PEETERS, 1994; MOUTQUIM *et al*., 1985). Essas alterações estão relacionadas à ação de estrógeno e progesterona os quais elevam a expressão do SRAA nos tecidos (COX *et al*., 1996), os níveis plasmáticos de renina (GANT N *et al*., 1973), de angiotensinogênio (GARLAND *et al*. 1987) e de angiotensina II (CALUWAERTS e*t al*., 2005).

A angiotensina II apresenta, na gravidez, elevada afinidade pelo receptor AT2 em detrimento do receptor AT1 (COX *et al*., 1996), resultando em vasodilatação e aumento do aporte de nutrientes para o feto. A angiotensina (1-7) também possui ação importante na gestação (LI et al., 1997), porquanto está relacionada com a liberação de óxido nítrico e prostaglandinas (CONRAD & BENYO, 1997), os quais estão envolvidos com a redução da pressão sanguínea materna.

SRAA e programação da função renal

O SRAA encontra-se ativado desde os tecidos intra-uterinos, incluindo a placenta como também no âmnio e côrion (KALLENGA e*t al*., 1996). No feto, o SRAA tem sido proposto como regulador do crescimento e da homeostase cardiovascular e renal (TUFRO-MCREDDIE e*t al*., 1995). Todos os componentes deste sistema são detectados no rim fetal de

ratos do 12º ao 17º dia de gestação e recém nascidos, apresentando concentrações maiores durante estas fases da vida em relação ao ser adulto (GOMES & NORWOOD, 1995).

Modificações na programação do SRAA estão relacionadas com alterações estruturais e funcionais na prole. Sódio elevado na dieta pode suprimir o SRAA. Assim, foi observado que ratos neonatos submetidos a uma sobrecarga de sódio durante a vida intra-uterina apresentam diminuição da afinidade da angiotensina II e de alguns componentes da matriz extracelular no córtex renal (BALBI *et al*., 2004). Quando mantidos em sobrecarga de sódio, ainda durante o aleitamento, estes animais apresentam lesões tubulares e intersticiais, glomeruloesclerose, redução do ritmo de filtração glomerular e albuminúria (MARIN *et al*. 2008).

Estresse oxidativo placentário e hipertensão materna

A presença moderada de espécies reativas de oxigênio (ROS) é necessária, em condições normais, para modular a expressão gênica sensível às reações de redox durante a gestação (DROGE, 2002; HANCOCK *et al*., 2001). Entretanto, o excesso de ROS pode levar a oxidação de proteínas estruturais, lipídios e de nucleotídeos da placenta e do feto (DROGE, 2002). O estresse oxidativo também pode modificar a estrutura e a função das proteínas celulares responsáveis pela sinalização celular e expressão gênica (BARFORD, 2004). A expressão de agentes inflamatórios como citocininas e TNF-α encontram-se elevada em placenta de mulheres com eclâmpsia (WANG & WALSH, 1996). Tal desordem está associada com estresse oxidativo elevado na circulação materna (BRAEKKE e*t al*., 2006). Ademais, estudo realizado em ratas que receberam alto teor de sódio na dieta, durante a última semana de gestação, mostrou elevação do estresse oxidativo em placentas (BEAUSEJOUR *et al*., 2007 a)

A hipertensão gestacional é considerada uma situação clínica de ação direta sobre o desenvolvimento fetal (GODFREY & BARKER, 2001), porquanto compromete os transportadores de aminoácidos, de glicose (JANSSON e*t al*., 2002) e o endotélio vascular na placenta (HUBEL , 1999; BROUGHTON, 1994). O aumento da atividade metabólica mitocondrial na placenta resulta em aumento do estresse oxidativo (WANG e*t al*., 1992; GIUGLIANO e*t al*., 1996), o qual pode favorecer a diminuição da disponibilidade de óxido nítrico (FRANCO & DANTAS, 2002), de forma a comprometer a resposta vasodilatadora do endotélio vascular (LENDA *et al*., 1999) na placenta. Alterações na vasculatura uterina e no fluxo sangüíneo placentário pode comprometer o crescimento fetal (LEANDRO *et al*., 2008).

A homeostase vascular na placenta é mantida através do equilíbrio dos prostanóides tromboxane A2 e prostaclina de modo a evitar agregação plaquetária e vasoconstrição (MONCADA & VANE, 1978). O estresse oxidativo elevado pode alterar esse equilíbrio a favor da vasoconstrição (WALSH *et al*., 1993). A redução do fluxo sangüíneo útero-placentário limita o suprimento nutricional para a placenta o que reduz o aporte nutricional no feto (BEAUSÉJOUR *et al.,* 2007 a).

Volume plasmático materno

Durante a gravidez normal ocorre uma gradual expansão de volume plasmático favorecida pela ativação do SRAA (CONRAD, 1984). O aumento dos níveis plasmático de aldosterona contribui para a retenção de sódio e água, resultando em expansão de volume da gravidez (LONGO, 1983; SCHRIER, 1991) que é acompanhada de redução da resistência periférica e de alterações da hemodinâmica cardiovascular (CONRAD & RUSS, 1992).

Fatores como a elevação do apetite para o sódio e o incremento da ingesta de água pelas mães estão relacionados com a expansão de volume (CHURCHILL e*t al*., 1980). Essas

mudanças comportamentais resultam em diminuição da osmolaridade plasmática durante a gravidez (ATHERTON e*t al*., 1982). Além disso, a síntese (ABBOUD *et al*., 1990; BERECEK & SWORDS, 1990) e a liberação de vasopressina (DAVISON *et al.,* 1988); DÜRR *et al.,* 1981) mostraram-se elevados em ratas grávidas

A alta ingestão materna de sódio pode induzir um meio intra-uterino hiperosmótico, condição que pode modificar a programação dos centros osmoreguladores na prole (NICOLAIDIS *et al*., 1990; CONTRERA & KOSTEN, 1983). Além disso, a supressão do SRAA materna pode comprometer o volume plasmático (BEAUSÉJOUR *et al.,* 2007a). A diminuição do volume de plasma circulante nas mães pode prejudicar o fluxo sanguíneo placentário e fetal de modo a prejudicar o crescimento *in utero* (REYNOLDS & REDMER, 1995: ROY-CLAVEL *et al.,* 1999).

2- JUSTIFICATIVA

O desenvolvimento de doença renal no ser adulto possui uma relação inversa com o crescimento intra-uterino. Durante esta fase a exposição a certos estímulos é capaz de alterar a programação fetal. Estudos epidemiológicos evidenciam que o ambiente intra-uterino inadequado pode programar alterações metabólicas e predispor o concepto ao desenvolvimento de doenças crônicas na idade adulta.

É sabido que a alta ingestão materna de sódio pode afetar o estresse oxidativo placentário e o volume plasmático que são fatores correlacionados com o desenvolvimento fetal. É também sabido que a alta ingestão materna de sódio, durante a gravidez e lactação, pode comprometer a homeostasia renal da prole na idade adulta. Portanto, avaliar se a sobrecarga de sódio no início da vida é capaz de levar a alterações na função renal do indivíduo adulto constitui uma forma de contribuir para o conhecimento da etiologia de doenças crônico-degenerativas..

3-OBJETIVO GERAL

Investigar: 1) se a ingestão materna de cloreto de sódio 0,17 M, em substituição da água de beber, a partir de vinte dias antes do acasalamento até o vigésimo dia de prenhez, afeta o volume plasmático e o estresse oxidativo placentário; 2) se esta ingestão materna de sódio é capaz de programar alterações na função renal da prole adulta; e, 3) se esta ingestão materna de sódio expandida durante a lactação afeta a função renal programada pelo tratamento pré-natal.

3.1 Objetivos Específicos

3.1.1 Estudo Materno

Avaliar, em mães mantidas com cloreto de sódio:

a) O estresse oxidativo placentário e o volume plasmático; b) o peso fetal da prole.

3.1.2 Estudo da prole adulta

Avaliar na prole de mães mantidas com cloreto de sódio:

a) O estresse oxidativo renal, triglicerídeos e colesterol séricos;

b) a pressão arterial média (PAM);

c) os parâmetros de hemodinâmica renal: fluxo sanguíneo renal (FSR), fluxo plasmático renal (FPR), filtração glomerular (FG), fração de filtração (FF) e resistência vascular renal (RVR); d) o número de néfrons, proteinúria e volume plasmático.

TRABALHO SUBMETIDO PARA PUBLICAÇÃO NO PERIÓDICO

PEDIATRIC NEPHROLOGY

Fetal development and renal function in adult rats prenatally subjected to sodium overload

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Abstract

Evidence points to an inverse relationship between prenatal growth and adult renal disease. This study evaluated factors correlated with fetal development, such as placental oxidative stress (Ox) and plasma volume (PV) of dams on sodium overload. Moreover, it evaluated the renal function of adult offspring from these dams. Wistar dams were maintained on a sodium chloride solution 0.17 M, instead drinking water, from twenty days before mating until either twentieth pregnancy day/parturition or weaning. Maternal and offspring tissue Ox, PV and 24h-urinary protein ($U_{Prot24h}$) were measured using colorimetric methods. The male offspring at age of 90 days had its renal hemodynamics evaluated using a blood pressure transducer, a flow probe and inulin clearance to measure respectively, mean arterial pressure (MAP), renal blood flow and glomerular filtration rate (GFR). The number of nephrons (NN) was counted in kidney suspension. Dams showed unchanged PV and placental Ox, as well as unchanged fetal weight, but increased $U_{Prot24h}$ (150%, $p<0.05$). Prenatally sodium overloaded rats presented increased $U_{Prot24h}$ (45%, $p<0.05$) and unchanged MAP, renal hemodynamics, NN and kidney Ox. Prenatally and postnatally sodium overloaded rats showed increased $U_{Prot24h}$ (27%, $p<0.05$) and kidney Ox (44%, $p<0.05$), reduced GFR (12%, $p<0.05$), increased PV (26%, $p<0.05$) and unchanged MAP and NN. Although just maternal proteinuria was changed and fetal growth retardation was not seen, prenatally sodium overloaded rats showed renal function alteration at adult life. Salt overload from prenatal to weaning leads to further renal function alterations than those seen in prenatal overloaded rats.

Keywords: fetal development . salt overload . renal blood flow . glomerular filtration rate . placenta . oxidative stress.

Introduction

Evidence has been pointing to an inverse relationship between prenatal growth and adult renal disease. Sodium chloride overload during perinatal period has been associated with renal dysfunction [1, 2] and changes in lipid metabolism at adult life [3].

Sodium overload may influence fetal development through the profile of plasma volume and the level of placental oxidative stress [4, 5], that in their turn influence placental blood flow [5]; and also through maternal dietary intake, since it has been shown that sodium chloride intake [6] or dehydration reduce dietary intake [7]. By these ways sodium overload may compromise fetal nutrition. Furthermore, sodium overload suppresses maternal plasma renin levels [6] and has been correlated with suppressed angiotensin II expression in the offspring kidney, during nephrogenesis [1]. The renin-angiotensin-aldosterone system (RAAS) seems fundamental for the nephrogenesis [8]. Variations in its level during fetal and first postnatal days of development may have tardy repercussion on renal function [8, 9].

Nephrogenesis in rats is completed around postnatal day 10 [10, 11], thus it may be hypothesized that maternal sodium overload during lactation period may perturb it, since the sodium may be transferred throughout the milk in rats [12]. In addition, the access of pups to drinking water, during the last days of lactation, might influence renal function at later life, considering that the window of programmation for adult RAAS might include this period [9].

It is known that maternal intake of sodium 0.3 M a sodium chloride solution increases placental oxidative stress [4, 5] and that maternal intake of sodium a sodium chloride solution 0.15 M, during pregnancy and lactation, affects the renal function of weanling rats [1]. In this study, it was investigated: 1) whether maternal intake of a sodium chloride 0.17 M as drinking water, from twenty days before mating until the twentieth pregnancy day, changes plasma volume and placental oxidative stress, factors correlated with fetal development that could be

predictive of later consequences to the offspring; 2) whether this maternal intake of sodium chloride programs renal function of offspring at adult life; and 3) whether this maternal sodium overload extended throughout lactation affects renal function programmed by prenatal treatment.

Material and Methods

Female Wistar rats, weighing 200 – 250 g, at age of 70 days, were randomly assigned. From then to parturition, the Control group (n=12) was maintained with tap water and the Saline group (n=15) was maintained with a sodium chloride solution 0.17 M, as drinking water. They were mated at age of 90 days; the presence of vaginal plug determined the 1st pregnancy day. Water balance was evaluated in dams on the 1st and 18th pregnancy day. Some dams of each group, Control (n=6) and Saline (n=8), had gestation interrupted on the 20th day for evaluation of plasma volume, hepatic and placental oxidative stress and some fetal parameters. Only placentas from male rats were weighed selected for evaluation of oxidative stress and only male fetuses were selected to weigh. Some dams (n=4) were maintained on saline up to weaning. The offspring maintained with tap water throughout the whole study were denominated Control group (C, n=16). The offspring maintained with saline (S) up to parturition (P) were denominated SP (n=16). And those maintained with saline during the prenatal and lactation (L) periods were denominated SPL (n=16). At birth, the litters were culled to 8 pups. After weaning, male offspring C, SP and SPL were maintained with tap water and diet *ad libitum*. At age of 90 days, half from each group were assigned to evaluation of plasma volume, oxidative stress and lipid metabolism studies. In the first hours of the morning, they were anesthetized using sodium pentobarbitone (Cristália Produtos Químicos Farmacêuticos, Itapira, SP, Brazil; 60 mg/kg, ip), for femoral artery catheterization. One blood sample was collected for measurement of postprandial serum triacylglycerols and

cholesterol. Plasma volume and renal oxidative stress were subsequently evaluated. The other halves of each group were assigned to evaluation of water balance, proteinuria, renal hemodynamics and nephron count. All the experimental procedures involving the animals described in this study were approved by the Committee for Ethics in Animal Experimentation of the Federal University of Pernambuco and carried out in accordance with the Committee guidelines.

Plasma volume measurement

Plasma volume was measured using the Evans Blue dye (Sigma-Aldrich Co., St. Louis, MO, USA) method as previously described [13-15], after anesthesia with sodium pentobarbitone (60 mg/kg, i.p.). Briefly, a femoral artery was catheterized and a basal blood sample, 1mL, was collected. Then the dye, 0.1%, prepared into physiological solution, was administered (100μg/100g body weight) through the femoral artery catheter. The catheter had previously been filled with physiological solution and after the dye administration it was flushed with 200 μl of physiological solution. After 7.5 min, the physiological solution in the catheter was discarded and a blood sample of 1mL was collected into a heparinized syringe. The blood was centrifuged in order to obtain the plasma sample. The dye content of the sample was determined spectrophotometrically at 610 nm (Spectrophotometer, UV-VIS RS 0223, LABOMED, Culver City, CA, USA) and compared to a standard curve designed from known amounts of Evans Blue dye and samples of basal plasma. In the sequence, the placentas, the fetuses and the livers were withdrawn.

Oxidative Stress Measurement

Tissue oxidative stress was evaluated using the thiobarbituric acid reactive substances (TBARS) levels according to the method of Buege & Aust, 1978 [16]. Each placenta or liver

was macerated in KCl (1.15%) at a proportion of 5mL/1g, during 15 min, in an ice bath and then transferred to test tubes. Afterwards, 1mL of the reagents, 0.375% thiobarbituric acid (Sigma-Aldrich) and 75% trichloroacetic acid (Vetec Química Fina Ltda., Rio de Janeiro, RJ, Brazil), were added to each milliliter of the tissue homogenate. Duplicate tubes for each reaction were sealed and heated in a water bath at a temperature of 100ºC for 15 min. After cooling, the protein precipitate was centrifuged for 10 min; then the supernatant was separated and the absorbance was measured at 535 nm.

Water balance and 24-h Proteinuria

Animals were placed in individual metabolic cages (Tecniplast Gazzada Sarl, Buguggiate, Italy). Diet and water intake were evaluated during a 24-hour period, and urine was collected for the same period. 24h urinary protein ($U_{Prot24h}$) was measured by precipitation with 3% sulfosalicylic acid [17].

Blood pressure and renal function in the adult offspring

Renal hemodynamics was measured after anesthesia with sodium pentobarbitone (60 mg/kg, i.p.), as previously described [14, 15]. Briefly, animals were tracheostomized and the left femoral artery, both jugular veins and the left ureter were catheterized. A flow probe (1.0 V, Transonic System, Ithaca, NY, USA) was placed around the left renal artery. Mean arterial pressure (MAP) and hematocrit (Hct) were measured immediately after femoral artery catheterization (initial MAP and initial Hct, respectively). In order to assess glomerular filtration rate (GFR), inulin (10% dissolved in physiological solution, 1.2ml/h) was infused through the left jugular vein and to maintain euvolemia**,** iso-oncotic serum (20% v/w) was infused through the jugular right vein, during surgery and throughout the evaluation of renal hemodynamics. After the surgical procedure was concluded, anesthesia was supplemented (45

mg/kg, i.p.). The evaluation of renal hemodynamics was initiated 1h after completion of surgery, and comprised two 20min intervals during which two urinary samples and three blood samples were collected. MAP and renal blood flow (RBF) were continuously monitored during both periods.

MAP and RBF were determined using a blood pressure transducer (Transpac, Abbot Laboratory, North Chicago, IL, USA) and a flow probe, respectively. MAP, RBF and heart rate (HR) recordings were analyzed by means of a playback program of the Calc Package Windaq. Renal plasma flow (RPF), filtration fraction (FF) and renal vascular resistance (RVR) were calculated according to the following equations: RPF = RBF x (1-Hct), FF = GFR/RPF, RVR = MAP/RBF. Each renal hemodynamic parameter was corrected according to the corresponding kidney weight (g).

The right kidney was used to determine the number of glomeruli, according to the method of Larsson [18]. Each kidney was sliced and incubated in 50% hydrochloric acid for 2h at room temperature. After mechanical dissociation, the homogenate was completed to 10mL with distilled water. Six aliquots of 30μL each were spread on glass slides over a surface of 4.5cm^2. Glomeruli were counted in every aliquot under a light microscope by two researchers in a blind fashion.

Analytical Methods

Inulin concentrations in urine and plasma were measured by the anthrone method [19]. Urinary Na^+ and K^+ excretion were measured using an electrolyte analyzer (Roche 9180 Electrolyte Analyzer, F. Hoffmann-La Roche Ltda., Basel, Suisse). Serum total cholesterol and triacylglycerols were estimated by the enzymatic method using commercial reagents (Labtest, Lagoa Santa, MG, Brazil).

Statistical analysis

The results were expressed as mean ± SEM. Differences between dams groups were analyzed using unpaired Student t test, while differences among offspring were analyzed using multiple comparison test Student-Newman-Keuls. Statmost 2.5 for Windows was used for statistical analysis (DataMost, Salt Lake City, UT, USA). The differences were considered significant at $P < 0.05$.

Results

Maternal data

Maternal data is shown on Table 1. Although, Saline dams showed, on the first day of pregnancy, body weight higher (8.2%, $p < 0.05$) than Control dams, the weight gain, during pregnancy, and body weight on the 20th day of pregnancy were similar between the two groups. Reproductive outcome, fluid intake and diet intake were also similar between the two groups. Water balance was similar between the two groups on the 1st day of pregnancy. However, on the 18th day of pregnancy, although urinary flow was higher (57%, $p < 0.05$) in Saline than in Control dams, water balance was higher in the former (53%, $p < 0.05$) than in the second group. The Control dams showed an important reduction (81%, $p < 0.05$) in sodium urinary excretion, from the 1st to the 18th day of pregnancy, the same was not seen in the Saline dams. Although water balance was higher in Saline dams on the 18th day of pregnancy, plasma volume was similar between the two groups on the 20th day of pregnancy. It was remarkable that $U_{Prot24h}$, on the 18th day of pregnancy, was higher in Saline dams (96%, $p < 0.05$) than in Control dams (Figure 1).

Data on placental corresponding to male fetuses and male fetuses is shown on Table 2. Placental weight and placental TBARS were the same for Saline and Control dams. Even hepatic TBARS, which was measured as a positive control, was the same for Saline and Control dams (7.7 ± 1.5 and 8.9 ± 2.5 mmol MDA/g tissue, respectively). Body weight and

kidney weight of male fetuses, as well as the ratio kidney weight/body weight were the same for both groups.

Offspring data

As was seen in the fetal weight, the birth weight was similar between SP and Control group (5.96 ± 0.11 and 6.31 ± 0.17 g, respectively). At 25 days old, when weaned, the body weight was similar among the three groups, SPL, SP and C (73 ± 2, 69 ± 2 and 69 ± 3 g, respectively). At age of 90 days, body weight of the SPL group was higher than that seen in C and SP groups (10 and 11%, respectively, $p < 0.05$, Table 3). Kidney weight was also higher in SPL compared with SP and C groups (14 and 9%, respectively, $p < 0.05$). $U_{Prot24h}$ was similar between SPL and SP and higher in both of them than in the C group (27 and 45%, respectively, $p < 0.05$, Figure 2). TBARS was higher in the kidney of SPL than in SP and C groups (35 and 44%, respectively, $p < 0.05$, Figure 3). The number of nephrons was the same for the three groups (Table 3). Urinary potassium excretion was higher in SPL and SP groups than in the C group (61 and 65%, respectively, $p < 0.05$), while plasma volume and urinary flow were higher in SPL than those values seen in C group (23 and 36%, respectively, $p < 0.05$). Non-fasting serum levels of triacylglycerols were in SP and SPL than those seen in C group (20% and 19%, respectively, $p < 0.05$), while serum cholesterol levels were similar between SP and C group, but higher in SPL than those values seen in SP and C groups (17% and 30%, respectively, $p < 0.05$).

Although MAP, RVR, and RBF (Table 4) were similar among the three groups, GFR was lower in SPL than in the SP and C groups (20 and 12%, respectively, $p < 0.05$, Figure 4). FF was also lower in SPL than in the SP group (24%, $p < 0.05$).

Discussion

Developmental origins of hypertension [20, 21] and renal disease [22] have gained evidence in the last years. Some reports have described an inverse relationship between prenatal growth and adult hypertension [23, 24] or renal disease [22]. In the present study, the sodium chloride overload, started twenty days before dams mating and maintained until the parturition, programmed proteinuria in the adult offspring, though it did not change neither fetal weight nor maternal parameters that could influence fetal development, such as plasma volume, placental oxidative stress and diet intake. Higher sodium overload than the presently used, such as 0.3 M, as drinking water, has been shown to increase placental oxidative stress [4] and to decrease maternal diet consumption [6]. Increased placental oxidative stress, through increase of thromboxane and decrease of nitric oxide levels [25, 26] leads to placental vasoconstriction, while low plasma volume leads to reduction in placental plasma flow [27]; and both conditions have been negatively correlated with fetal weight. In the present study, even the hepatic maternal oxidative stress, measured as a positive control, was unchanged. Together the unchanged placental oxidative stress, maternal plasma volume, diet intake and gain weight, suggest that fetal nutrition was not compromised. These parameters were emphasized taking in account that undernutrition during pregnancy has been correlated with low birth weight and compromised cardiovascular [28] and renal function [29] at adult life. Except for the increased maternal proteinuria, no other predictive sign of late repercussion was detected among the maternal parameters studied, although the possibility of maternal edema may be considered, since at 18^{th} pregnancy day the water balance was elevated in the dams treated with saline.

Increased proteinuria in the prenatally sodium overloaded rats was independent of the number of nephrons and renal hemodynamic profile that were unchanged. Although

albuminuria had not been measured, the increased proteinuria may include albuminuria. Considering this assumption, microalbuminuria has been pointed not only as a marker for renal disease, but as an early marker for cardiovascular disease in healthy individuals [30]. Like in the present work, Porter and coworkers [31], limiting high sodium intake to prenatal period, have shown that the adult animals did not present increased blood pressure; though when these animals were exposed to stress they presented augmented blood pressure. Another disturbance showed by the animals prenatally treated was increased serum triacylglycerols and increased kaliuresis. Different from our finding, Vidonho and coworkers [3] have shown reduction of fasting triacyglycerols in the offspring of dams maintained on an 1.36M dietary salt overload, during pregnancy. Two factors may explain the contrast, one is the content of sodium consumed and the other is the fact that blood had been collected after fasting, different from the present study. Although conflicting, this evidence shows that prenatal sodium overload imprint changes in lipid metabolism. The increased kaliuresis suggest that aldosterone is increased in these animals. Furthermore, there is evidence that angiotensin II is increased in the kidney of adult rats submitted to high dietary sodium during prenatal life [32].

Nephrogenesis was not affected either by prenatal or prenatal plus postnatal sodium overload. Similarly, rats submitted to prenatal sodium chloride 0.15 M from prenatal period to weaning, did not show, at age of 30 days, alteration in the number of nephrons [1]. Maternal intake of sodium chloride 0.15 M, during pregnancy, has shown to reduce maternal plasma renin activity and plasma aldosterone levels [6]. This reduction in maternal RAAS may lead to diminished angiotensin II expression in the kidney of the offspring during nephrogenesis [1, 8]. However, it has not been associated with reduced number of nephrons, different from pharmacological blockade of AT1 receptor that compromises nephrogenesis [8].

Independent of the number of nephrons, the reduction in GFR shown by rats maintained on sodium overload during prenatal and also during lactation, was also independent of other changes in renal hemodynamics. This profile is compatible with a reduction in the filtration area. Pups at age of 30 days that were maintained during pregnancy and lactation on sodium overload 0.15M also has shown reduced GFR [1]. Our data indicates that the increased oxidative stress was irreversible programmed in the kidney of these animals during the lactation. They presented increased body weight at age of 90 days, which was likely related to the observed increased plasma volume and dyslipidemia, since they did not show increased diet intake, but showed elevated serum triacyglycerols and cholesterol. Aside from occurring due to a possible increment in sodium retention, the increment in plasma volume may also occur due to a reduction in sodium filtration. It appears controversial increased plasma volume associated with increased urinary flow and unaltered fluid intake, such as these rats exhibited, nevertheless distal handling of water is dependent on arginine vasopressin that is known to present a higher plasma osmolality threshold for secretion of this hormone [33, 34]. The additional alterations shown by the group maintained on sodium overload until weaning compared with the group maintained until birth, reinforces evidence that renal functional programmation continues to occur during the first postnatal weeks [9].

In summary, the present results show that prenatal salt overload-induced changes in lipid metabolism and renal function were not associated with fetal growth retardation. They were associated only with a silent early symptom, the maternal proteinuria. Furthermore, these results show that maintenance of sodium overload during lactation exacerbates renal dysfunction programmed during intrauterine period, reinforcing evidence that renal function continues to be programmed during this period.

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Table 1 Maternal data

	Control (n=6)	Saline (n=8)
Body weight, 1st day, g	241 ± 3	261 ± 8*
Body weight, 20th day, g	336 ± 9	362 ± 11
Reproductive outcome	11.8 ± 0.7	13.2 ± 0.5
Gain weight, g	96 ± 10	95 ± 13
Fluid intake, 1st day, mL/100 g	19 ± 4	27 ± 2
Urinary flow, 1st day, mL/100 g	7 ± 2	12 ± 2
Water balance, 1st day, mL/100 g	9 ± 3	14 ± 3
Fluid intake, 18th , day, mL/100 g	19 ± 1	30 ± 5
Urinary flow, 18th day, mL/100 g	7 ± 1	11 ± 1*
Water balance, 18th day, mL/100 g	13 ± 1	20 ± 2*
Diet intake, 1st day, g/100 g	8 ± 1	7 ± 1
Diet intake, 18th , g/100 g	9 ± 1	8 ± 1
$U_{Na+}V$, 1st day, mmol/100 g/24 h	1.43 ± 0.64	2.36 ± 0.45
$U_{Na+}V$, 18th day, mmol/100 g/24 h	0.26 ± 0.43	2.12 ± 0.65*
$U_{K+}V$, 1st day, mmol/100 g/24 h	1.16 ± 0.23	1.18 ± 0.19
$U_{K+}V$, 18th day, mmol/100 g/24 h	1.38 ± 0.22	1.16 ± 0.26
Plasma volume, 20th day, mL/100 g	4.5 ± 1	4.2 ± 1

Control, dams maintained with drinking water and Saline, dams maintained, from age of 70 days to parturition, with sodium chloride 0.17M. 1st, 18th and 20th are pregnancy days. $U_{Na+}V$ is urinary sodium excretion. $U_{K+}V$ is urinary potassium excretion. Results are mean ± SEM. $P<0.05$ vs. Control (Students unpaired "t" test).

Table 2 Data on placenta and male fetuses on the 20th day of pregnancy.

	Control (n=6)	Saline (n=8)
Placental weight, g	0.46 ± 0.01	0.44 ± 0.02
Placental TBARS, mmol MDA/g tissue	7.5 ± 1.5	8.4 ± 2.3
Body weight fetuses, g	2.8 ± 0.2	2.8 ± 0.3
Kidney weight fetuses, mg	18 ± 2	16 ± 3
Kidney weight/body weight fetuses, %	0.67 ± 0.04	0.57 ± 0.05

N represents the mean of litters evaluated. 50 placentas and fetuses were evaluated in Control and 58 were evaluated in Saline group. Control, dams maintained with drinking water and Saline, dams maintained, from age of 70 days to parturition with sodium chloride 0.17M. MDA means malonyldialdehyde. Results are mean ± SEM.

Table 3 General data seen in offspring at age of 90 days

	C (n=8)	SP (n=8)	SPL (n=8)
Body weight, g	346 ± 11	348 ± 9	383 ± 8*†
Kidney Weight, g	1.39 ± 0.04	1.32 ± 0.2	1.51 ± 0.04*†
Kidney Weight/Body Weight, %	0.37 ± 0.01	0.35 ± 0.01*	0.39 ± 0.01†
Number of Nephrons	49300±2931	48666±2733	53000±2766
Fluid intake, mL/100 g	11 ± 1	12 ± 1	11 ± 1
Diet intake, g/100 g	6.0 ± 0.6	5.6 ± 0.7	6.5 ± 0.3
Urinary flow, mL/100 g	2.8 ± 0.4	3.3 ± 0.2	3.8 ± 0.3*
$U_{Na+}V$, mmol/100 g/24 h	0.29 ± 0.04	0.31 ± 0.03	0.35 ± 0.04
$U_{K+}V$, mmol/100 g/24 h	0.84 ± 0.13	1.39 ± 0.14*	1.35 ± 0.09*
Plasma volume, mL/100 g	2.97 ± 0.25	3.35 ± 0.30	3.74 ± 0.40*
Triacylglycerols, mmol/dL	0.82 ± 0.03	0.99 ± 0.09*	0.98 ± 0.06*
Cholesterol, mmol/dL	1.40 ± 0.15	1.56 ± 0.10	1.82 ± 0.05*†

C is offspring from dams maintained with drinking water, SP is offspring from dams maintained with sodium chloride up to parturition, and SPL is offspring from dams maintained with sodium chloride during pregnancy and lactation. $U_{Na+}V$ is urinary sodium excretion, $U_{K+}V$ is urinary potassium excretion. Results are mean ± SEM. $P<0.05$: * vs. Control, † vs. SP (Student-Newman-Keuls).

Table 4: Blood pressure and renal hemodynamics seen in the offspring at age of 90 days

	C (n=8)	SP (n=8)	SPL (n=8)
MAP, mmHg	111 ± 1	112 ± 4	112 ± 3
Hct	0.49 ± 0.01	0.50 ± 0.01	0.48 ± 0.01
RVR, mmHg/mL/min/g	21.70 ± 1.84	23.16 ± 3.42	23.62 ± 1.39
RBF, mL/min/g	5.40 ± 0.70	5.40 ± 0.50	4.92 ± 0.30
RPF, mL/min/g	2.72 ± 0.38	2.63 ± 0.28	2.52 ± 0.20
FF	0.37 ± 0.03	0.42 ± 0.06	0.32 ± 0.01†

C is offspring from dams maintained with drinking water, SP is offspring from dams maintained with sodium chloride up to parturition, and SPL is offspring from dams maintained with sodium chloride during pregnancy and lactation.

Figure Legends

Figure 1: 24h urinary protein. Control, dams maintained with drinking water and Saline, dams maintained, from age of 70 days up to parturition, with sodium chloride 0.17M. Results are mean ± SEM. * P<0.05 vs. Control (Students unpaired "t" test).

Figure 2: 24h urinary protein. C is offspring from dams maintained with drinking water, SP is offspring from dams maintained with sodium chloride up to parturition, and SPL is offspring 90 days from dams maintained with sodium chloride during pregnancy and lactation. Results are mean ± SEM.*P<0.05 vs. Control (Student-Newman-Keuls).

Figure 3: TBARS is thiobarbituric acid reactive substances**.** MDA means malonyldialdehyde. C is offspring from dams maintained with drinking water, SP is offspring from dams maintained with sodium chloride up to parturition, and SPL is offspring from dams maintained with sodium chloride during pregnancy and lactation. Results are mean ± SEM. P<0.05: * vs. Control, † vs. SP (Student-Newman-Keuls).

Figure 4: Glomerular filtration rate (GFR). C is offspring from dams maintained with drinking water, SP is offspring from dams maintained with sodium chloride up to parturition, and SPL is offspring from dams maintained with sodium chloride during pregnancy and lactation. Results are mean ± SEM. P<0.05: * vs. Control, † vs. SP (Student-Newman-Keuls).

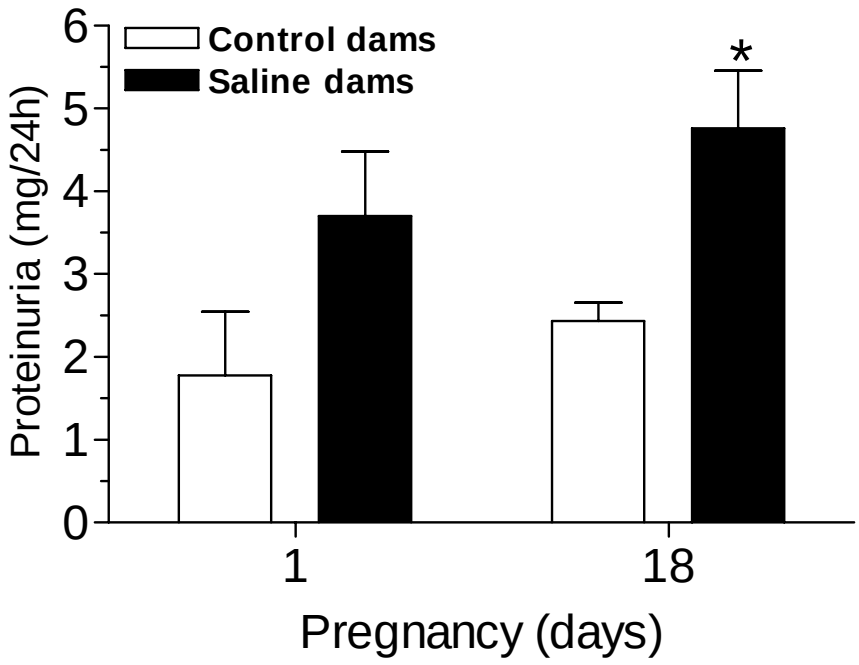
Control dams
Saline dams
Proteinuria (mg/24h)
0
1
2
3
4
5
6
*
1
18
Pregnancy (days)

Figure 1

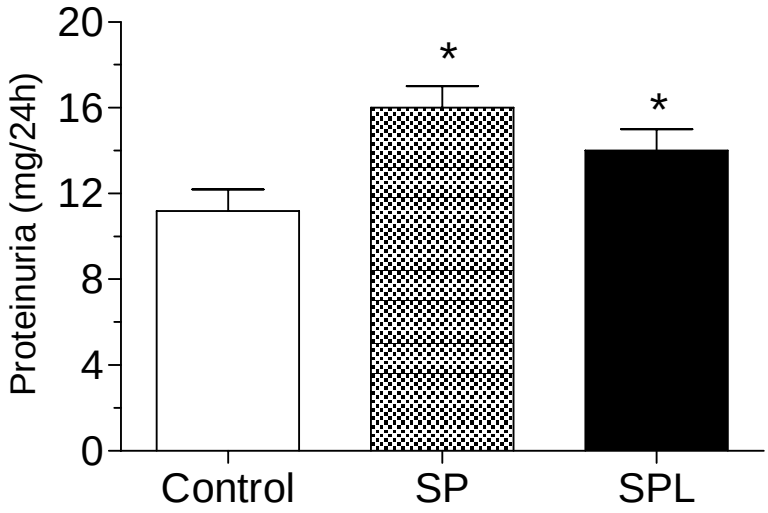

Figure 2

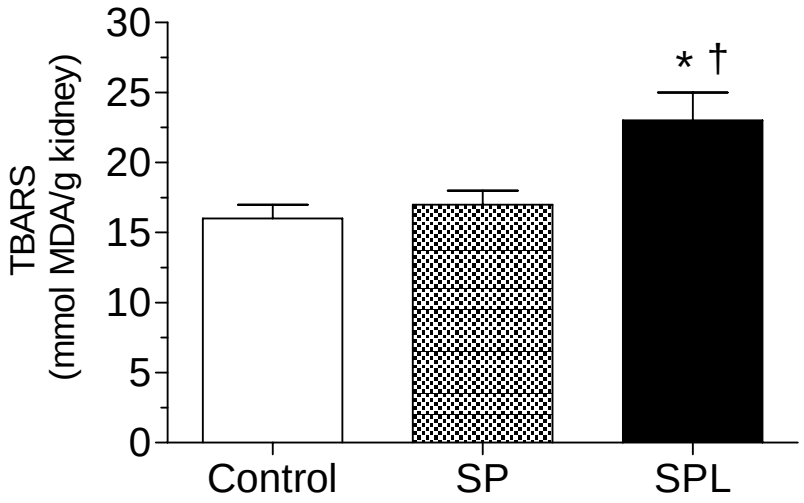

Figure 3

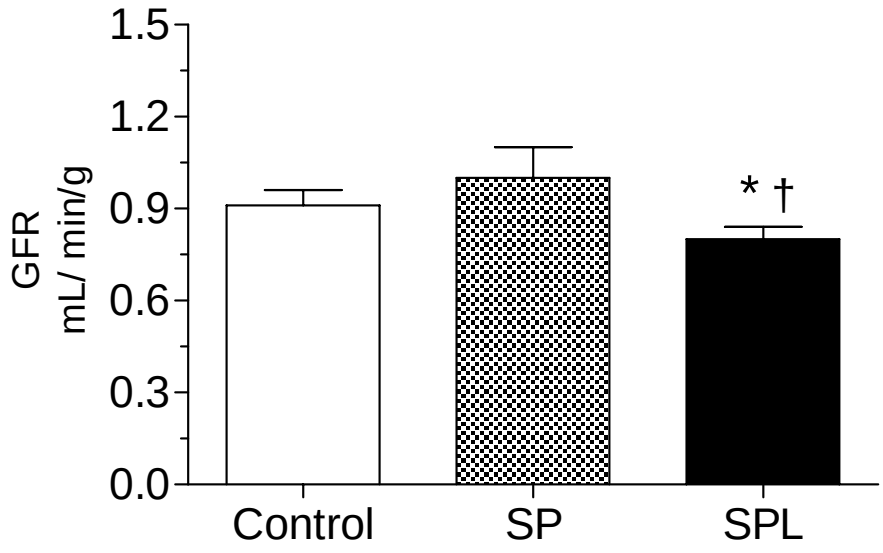

Figure 4

5-CONCLUSÕES

- A sobrecarga de sódio materna, produzida no presente trabalho, não afetou o peso fetal nem parâmetros maternos diretamente correlacionados com o desenvolvimento fetal. Apenas a proteinúria materna apresentou-se elevada;
- A sobrecarga de sódio pré-natal programou alterações na função renal e no metabolismo lipídico na prole adulta;
- A manutenção da sobrecarga de sódio durante a lactação exacerbou a disfunção renal. Este dado reforça evidências de que a função renal continua a ser programada durante o período pós-natal.

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ANEXO

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Ofício nº 42/07

Recife, 21 de maio de 2007

Da Comissão de Ética em Experimentação Animal (CEEA) da UFPE
Para: **Profa. Ana Durce Oliveira da Paixão**
Departamento de Fisiologia e Farmacologia - UFPE
Processo nº 004933/2007-72

Os membros da Comissão de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEEA-UFPE) avaliaram a resposta de V. Sa. referente ao primeiro parecer da CEEA sobre o projeto de pesquisa intitulado **"Avaliação do perfil metabólico e da função renal em ratos adultos submetidos à sobrecarga de sódio durante a vida intra-uterina".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEEA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 9.605 – art. 32 e Decreto 3.179-art 17, de 21/09/1999, que trata da questão do uso de animais para fins científicos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais realizados.

Atenciosamente,

Prof. Silene Carneiro do Nascimento
UFPE
Presidente CEEA