UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

Moisés Diôgo de Lima

Análise dos mecanismos fisiopatológicos relacionados ao parto pré-termo: corioamnionite histológica, estresse oxidativo e apoptose.

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho", para obtenção do título de Doutor em Patologia.

Orientadora: Profª Drª Márcia Guimarães da Silva

Botucatu
2016

Moisés Diôgo de Lima

Análise dos mecanismos fisiopatológicos relacionados ao parto pré-termo: corioamnionite histológica, estresse oxidativo e apoptose.

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho", para obtenção do título de Doutor em Patologia.

Orientadora: Profª Drª Márcia Guimarães da Silva

Botucatu
2016

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Lima, Moisés Diôgo.
Análise dos mecanismos fisiopatológicos relacionados ao parto pré-termo : corioamnionite histológica, estresse oxidativo e apoptose / Moisés Diôgo Lima. - Botucatu, 2016

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu
Orientador: Márcia Guimarães da Silva
Capes: 40105008

1. Prematuros. 2. Corioamnionite. 3. Stress oxidativo. 4. Apoptose. 5. Gravidez - Complicações.

Palavras-chave: Apoptose; Corioamnionite; Estresse oxidativo; Infecção intra-amniótica; Prematuridade.

Epígrafe

“Não existe verdade real nesse mundo apenas a percepção de cada um sobre ela”

Pensamento egípcio

Dedicatória

Dedico esse singelo trabalho à minha família; alicerce da minha existência.

Agradecimentos

Agradeço, sinceramente,

À Maria do Socorro Costa, secretária da Unidade Materno-infantil do Hospital Universitário Lauro Wanderley / Universidade Federal da Paraíba pela dedicação com que sempre me atendeu;

Aos meus colegas de trabalho da Unidade Materno-infantil do Hospital Universitário Lauro Wanderley / Universidade Federal da Paraíba pelo apoio constante no acompanhamento clínico das mulheres envolvidas nessa pesquisa;

Ao Professor Alexandre Rolim do Departamento de Cirurgia do Centro de Ciências Médicas / Universidade Federal da Paraíba pela gentileza em nos assessorar no laboratório de anatomia patológica do Hospital Universitário Lauro Wanderley / Universidade Federal da Paraíba;

Aos colegas de turma do programa DINTER - UNESP/ UFPE pela simpatia dos nossos encontros;

À coordenação do Programa de Dinter UNESP/UFPE pela iniciativa em proporcionar essa oportunidade. À secretária do Programa de Pós-Graduação em Patologia, UNESP, pela atenção dedicada à parte burocrática do processo de Pós-Graduação;

À Capes, pelo financiamento do Programa Dinter UNESP/UFPE;

Aos colegas Professores do Departamento de Obstetrícia e Ginecologia do Centro de Ciências Médicas da Universidade Federal da Paraíba pelo incentivo;

Em especial à Laura Fernandes Martin e aos demais pós-graduandos do Laboratório de Imunopatologia da Relação Materno Fetal do Deapartamento de Patologia da Faculdade de Medicina de Botucatu, UNESP, pela contribuição em momentos cruciais desse trabalho;

À Professora Doutora Márcia Guimarães da Silva pela competente e amigável orientação acadêmica.

Resumo

O parto pré-termo espontâneo, definido como nascimento antes das 37 semanas completas de gestação apesar dos consideráveis avanços científicos ainda representa um importante problema médico, humano e social. É representado por dois grandes grupos de patologias obstétricas definidas como Trabalho de Parto Prematuro (TPP) e Rotura Prematura de Membranas Pré-Termo (RPM-PT). O primeiro objetivo deste estudo foi avaliar os níveis de 8-oxo-2'-desoxiguanosina (8-OHdG) em membranas corioamnióticas de gestações complicadas pela prematuridade. Neste estudo transversal, foram estudadas 31 membranas corioamnióticas de gestações complicadas por TPP e 35 com RPM-PT cujos partos ocorreram no período compreendido para a prematuridade. Como grupo controle foram incluídas 37 membranas corioamnióticas de partos a termo. As membranas corioamnióticas foram coletadas e a extração total de DNA foi realizada pelo Kit genomic-Prep Mini Spin e os níveis de 8-OHdG níveis foram analisados pela técnica de ELISA empregando-se o kit Highy Sensitive 8-OHdG. Em relação aos dados obtidos, os níveis de 8-OHdG (2,90 ng/mL [1,54 – 4,06]) nas membranas corioamnióticas do grupo termo foram significativamente maiores que no grupo de partos prematuros (0,61ng/mL [mínimo: 0,37 – máximo: 0,92]) (p <0,0001). Os níveis de 8-OHdG foram também mais elevados no grupo de termo do que nos grupos PTL (0,71 ng/mL [mínimo: 0,40 – máximo:1,47]) ou RPM-PT (0,53 [mínimo: 0,37 – máximo: 0,71]) (p <0,0001), respectivamente. Nossos dados reforçam a tese que os danos oxidativos estão presentes nas membranas corioamnióticas de gestações a termo como consequência de um processo fisiológico do envelhecimento dos tecidos gestacionais. O segundo objetivo deste estudo foi analisar a associação entre corioamnionite histológica, ocorrência de apoptose e níveis de 8-OHdG em membranas corioamnióticas de gestações complicadas por RPM-PT e TPP. Foi realizado um estudo prospectivo e um total de 60 gestantes foram recrutadas, sendo 31 gestações complicadas por RPM-PT e 29 com TPP. Após o parto, as membranas corioamnióticas foram submetidas ao exame histopatológico, à análise dos níveis de 8-OHdG por ELISA, empregando-se o kit Highy Sensitive 8-OHdG, e quanto a ocorrência de apoptose pela imunomarcação da proteína p53. A corioamnionite histológica foi detectada em 64,5% das membranas corioamnióticas do grupo RPM-PT e em 44,8% do grupo TPP (p = 0,12). A ocorrência de apoptose no grupo RPM-PT (32,2%) foi semelhante no grupo TPP (24,1%) (p=0,48). Os níveis de 8-OHdG no grupo TPP [0,71 (mínimo: 0,43 – máximo: 1,38)] foram significativamente maiores do que no grupo RPM-PT [0,53 (mínimo: 0,37- máximo: 0,69)] (p = 0,02). A análise de regressão logística entre esses mecanismos demonstra que apenas a corioamnionite histológica está relacionada à apoptose (OR = 3,7; IC 95% = 1,19-14,9; p = 0,04). Concluimos que entre os fatores analisados a corioamnionite e a imunomarcação da proteína p53 foram mais prevalentes no grupo de mulheres acometidas pela RPM-PT, que os níveis de 8-OHdG foram mais elevados no grupo de

mulheres acometidas pelo TPP e que entre estes fatores a corioamnionite histológica parece estar mais claramente associada à ocorrência de apoptose em membranas corioamnióticas de gestações complicadas por prematuridade espontânea. O terceiro objetivo deste estudo foi analisar a associação de desfechos neonatais adversos, especialmente a sepse neonatal precoce, em gestações complicadas por RPM-PT e TPP na presença de corioamnionite histológica. Foi realizado um estudo caso-controle e um total de 73 gestantes foram incluídas no estudo, sendo 39 gestantes com RPM-PT e 34 com TPP. Após o parto, as membranas foram submetidas ao exame histopatológico e os recém-nascidos foram avaliados para o diagnóstico de eventos adversos: escore de Apgar <7 aos 5 min e 10 min, admissão em unidade de terapia intensiva neonatal, dificuldade respiratória, intubação traqueal e sepse neonatal precoce (SNP), esta última definida como sendo uma síndrome clínica observada nas primeiras 72 h de vida do recém-nascido. Em ambos os grupos as características maternas e neonatais (idade materna, índice de massa corpórea, etnia, paridade, idade gestacional e peso do recém-nascido) não foram significativamente diferentes. Entre as variáveis analisadas no contexto da prematuridade observamos que a SNP foi a mais prevalente no grupo RPM-PT e corioamnionite distinguindo-se estatisticamente (p = 0,04). Os outros desfechos neonatais adversos não mostraram diferença estatística entre si ou entre as patologias obstétricas envolvidas na pesquisa. No modelo de regressão logística, verificou-se que a presença de corioamnionite histológica (OR = 7,4, IC 95%: 1,95-35,9, p =0,0005), a dificuldade respiratória (OR = 3,5; IC 95%: 1,02-12,9; p = 0,049) e RPM-PT (OR = 9,8; IC95%: 2,95-38,5; p = 0,0004), entre as variáveis independentes, influenciaram diretamente a ocorrência de SNP. A análise da curva ROC reforça a tese que a corioamionite e a RPM-PT são critérios relevantes para o evento de SNP com índices de sensibilidade de 76% e especificidade de 82%, respectivamente. Conclui-se que a corioamnionite histológica e a ocorrência de RPM-PT estão associadas a desfechos neonatais adversos, especialmente a ocorrência de sepse neonatal precoce.

Palavras-chave: Prematuridade, corioamnionite histológica, estresse oxidativo e apoptose.

Abstract

Spontaneous preterm birth, defined as birth before 37 weeks completed gestation, despite the considerable scientific advances, still represents an important medical problem, human and social. It is represented by two large groups of obstetric pathologies defined as preterm labor (PTL) and premature rupture of preterm ovular membranes (pPROM). The first objective of this study was to evaluate the levels of 8-oxo-2'-deoxiguanosine (8-OHdG) in amniochorion membranes from pregnancies complicated by prematurity. In this cross-sectional study, were enrolled 31 with PTL and 35 with pPRM who presented preterm delivery. As control group was included 37 pregnant women that delivery at term. Amniochorion membranes were collected and total DNA extraction was performed by ILLUSTRA tissue & cells genomic-Prep Mini Spin Kit and 8-OHdG levels were measured by an ELISA Highy Sensitive 8-OHdG Check kit. Regarding to data, 8-OHdG levels in amniochorion membranes of term group (2,90 ng/mL [min: 1,54 – max:4,06]) were significantly higher than premature group (0,61 ng/mL [min: 0,37 – max:0,91]) ($p<0.001$). 8-OHdG levels were also higher in term group than in PTL (0,71 ng/mL [min: 0,40 – max:1,47]) or pPROM groups (0,53 ng/mL [min: 0,37 – max:0,71]) ($p<0.001$), respectively. Our data reinforces that oxidative damage are present at term pregnancies as physiologic process of amnionchorion aging. The second objective of this study was to analyze the association among histologic chorioamnionitis, apoptosis occurence and 8-OHdG levels in amniochorion membranes from pregnancies complicated by pPROM and PTL. It was a prospective study and a total of 60 pregnant women were enrolled, being 31 pregnant women who presented pPROM and 29 with PTL. After delivery, the amniochorion membranes were subjected to a histopathological examination, to 8-OHdG levels analysis by an ELISA Highy Sensitive 8-OHdG Check kit and occurrence of apoptosis by p53 protein immunohistochemical study. Histologic chorioamnionitis was detected in 64.5% of the amniochorion membranes from pPROM group and in 44.8% from PTL group ($p=0.12$). The occurence of apoptosis in the pPROM group (32.2%) was similar in the PTL group (24.1%) ($p=0.48$). The 8-OHdG levels in the PTL group [0.71 (0.43-1.38)] were significant higher than in the pPROM group [0.53 (0.37-0.69)] ($p=0.02$). Logistic regression analysis among these mechanisms showed that only histologic chorioamnionitis is related to apoptosis (OR=3.7; 95%CI= 1.19-14.9; $p=0.04$). Histologic chorioamnionitis seems to be more clearly associated with apoptosis occurrence in amniochorion membranes from pregnancies complicated by spontaneous prematurity. The third objective of this study was to analyze the association of adverse neonatal outcomes, especially early neonatal sepsis (ENS), in pregnant women affected by pPROM and PTL in the presence of histologic chorioamnionitis. It was a case-control study and a total of 73 pregnant women were enrolled, being 39 pregnant women who presented pPROM and 34 with PTL. After delivery, the membranes were subjected to a histopathological examination

and the newborn infants were evaluated for the diagnosis of adverse events: Apgar score <7 at 5 min and 10 min, admission to a neonatal intensive care unit, respiratory distress, use of continuous positive airflow pressure (CPAP), tracheal intubation and early neonatal sepsis, defined as being a clinical syndrome observed in the first 72 h of life of the newborn. In both groups, the sample general characteristics (maternal age, body mass index, ethnicity, parity, gestational age, and weight of the newborn) were not statistically different. Except ENS, which was the most prevalent in the pPROM and chorioamnionitis groups (p=0.04), the other neonatal adverse outcomes showed no statistical difference between them or between obstetric pathologies involved in the research. In a logistic regression model, we found that the presence of histologic chorioamnionitis (OR=7.4; 95%CI=1.95-35.9; p=0.0005), respiratory distress (OR=3.5; 95%CI=1.02-12.9; p=0,049) and pPRM (OR=9.8; 95%CI= 2.95-38.5; p=0.0004) among the independent variables, directly influenced ENS occurrence. ROC analysis reinforced that choriamnionites and pPROM are as criteria of relevant sensitivity (76.0%) and specificity (82.0%) for the ENS event. We conclude that histological chorioamnionitis and the occurrence of pPROM are associated with adverse neonatal outcomes, especially the occurrence of early neonatal sepsis.

Keywords: Prematurity, histologic chorioamnionitis, oxidative stress, apoptosis.

Lista de abreviaturas

AP-1	Proteína Ativadora – 1
O_2^-	Ânion superóxido
CAM	Corioamnionite
COX-2	Ciclooxigenase – 2
Cu^{2+}	Cátion Cobre
DNA	Ácido desoxirribonucleico
EROs	Espécies reativas de oxigênio
ERNs	Espécies reativas de nitrogênio
FADD	Fas associado ao domínio de morte
FasL	Ligante para a proteína Faz
Fe^{2+}	Cátion ferro
H_2O_2	Peróxido de hidrogênio
IIA	Infecção intra-amniótica
IFN	Interferon
IL	Interleucina
INF-α	Interferon alfa
INF-β	Interferon beta
IRF3	Fator regulador de interferon 3
IRF7	Fator regulador de interferon 7
kDa	Quilo Dalton
MDA	Malonaldeído
MEC	Matriz Extracelular
MMP-2	Metaloproteinase-2
NF-kB	Fator nuclear kB
OGG1	Oxoguanina glicosilase
PAMPs	Padrões moleculares associados a patógenos
$ONOO^-$	Peroxinitrito
P38MAPK	Proteinoquinase ativada por mitógeno
Ras-GTPase	Trifosfato guanosina hidroxilase
RL	Radical livre
OH^-	Radical hidroxil
NO_2	Radical nitro
RPMO-PT	Rotura prematura de membranas ovulares pré-termo
TNF-α	Fator de necrose tumoral alfa
TBAR	Reagente do ácido tiobarbitúrico
TIMP	Fator tecidual inibidor de metaloproteinase
TIR	Receptor Toll/IL -1
TLR	Toll-like receptor
TPP	Trabalho de parto prematuro
TRADD	Receptor TNF associado ao domínio de morte
8-OHdG	8-hidroxi-2'-deoxiguanosina
3-NT	3-nitrotirosina

Sumário

Sumário

Capítulo I

Revisão da Literatura

1.1. Prematuridade: conceito, incidência e fatores de risco

A prematuridade, conceitualmente definida como o nascimento antes de completadas as 37 semanas de gestação, não obstante os consideráveis avanços científicos no conhecimento da fisiologia do parto e dos significativos progressos tecnológicos que ocorreram na medicina perinatal nas últimas décadas, ainda representa um problema médico, humano e social a ser enfrentado (Liu et al., 2012).

O parto pré-termo pode ocorrer por indicações médicas, portanto, artificialmente induzido, ou de forma espontânea. O primeiro, devido a comprometimento materno/fetal, incluindo desordens hipertensivas, diabetes, doença cardíaca, patologias placentárias, malformações fetais, restrição de crescimento fetal e o segundo, representado por dois grandes grupos de patologias obstétricas definidas como: o Trabalho de Parto Pré-Termo (TPP), quando a gestante desencadeia precocemente as contrações uterinas associadas a modificações morfológicas da cérvice como o esvaecimento e dilatação do colo uterino, mantendo, entretanto, a integridade das membranas corioamnióticas e a Rotura Prematura de Membranas Pré-Termo (RPM-PT), entidade clínica caracterizada pela rotura das membranas fetais e expulsão do líquido amniótico contido nelas sem o desencadear de contrações uterinas (Corrêa et al., 2002).

A taxa de incidência da prematuridade é variável e tem permanecido estável ao longo de décadas na maioria dos países em desenvolvimento oscilando entre 5 a 18% entre todas as gestações estimando-se, globalmente, cerca de 15 milhões de novos recém-nascidos prematuros a cada ano (Blencowe et al., 2012). Sobre o nascimento prematuro sabe-se, ainda, que aproximadamente 70% deles ocorrem de forma espontânea e resultam do TPP (45%) e da RPM-PT (25%). Os demais 30% são atribuídos à interrupção eletiva da gestação por intercorrências clínicas maternas e/ou fetais (Muglia et al.,2010).

É relevante salientar que cerca de 5% dos recém-nascidos pré-termo nascem com menos de 28 semanas de gestação (prematuridade extrema), 15% nascem entre 28-31 semanas (prematuridade severa), 20% são aqueles nascidos entre 32–33 semanas

(prematuridade moderada) e 60–70% nascem com 34–36 semanas (prematuridade tardia) o que torna o nascimento prematuro um desafio clínico, uma vez que, está fortemente relacionado a complicações maternas e principalmente neonatais, imediatas e a longo prazo, o que, por si só, representa importante problema de saúde pública (Goldenberg et al., 2008).

No âmbito das sequelas advindas do nascimento pré-termo há relatos de mulheres que, passando por essa experiência, estão mais propensas ao parto cirúrgico, à depressão pós-parto e à longa permanência de internação hospitalar (Hamilton et al., 2010). Os neonatos, por sua vez, estão mais sujeitos a problemas súbitos como estresse respiratório, enterocolite necrotizante, hemorragia intraventricular, retinopatias e dificuldades alimentares, e a longo prazo, inabilidades de ordem visual, motora e de aprendizagem além de doenças pulmonares crônicas e elevado índice de infertilidade na vida adulta (Avchen et al., 2001; Swamy et al., 2008).

Múltiplos são os fatores de risco relacionados ao nascimento pré-termo. Suas causas diferem entre si, o que torna ainda mais desafiador o seu entendimento etiopatogênico, principalmente porque que o fenótipo mais comumente encontrado é o do nascimento prematuro espontâneo em grande parte relacionado às mulheres de baixo risco para a prematuridade (Goldenberg et al., 2008). Entre os estudos epidemiológicos de caráter populacional e com análise de grupos étnicos sabe-se que o TPP é mais comum entre as mulheres brancas, enquanto que a RPM-PT é mais incidente entre as autodeclaradas negras. Essa variabilidade tem sido motivo para diferentes interpretações em diversos estudos (Ananth et al.,2006) e admite-se que os fatores genéticos e epigenéticos passam a ser cada vez mais reconhecidos como os grandes determinantes do parto pré-termo, embora, a magnitude do seu efeito e do seu grau, ainda permaneçam indefinidos (Muglia et al.,2010).

Pesquisas relacionadas aos mecanismos que envolvem a etiopatogenia da prematuridade têm apontado para um entendimento fisiopatológico que, embora multifatorial, convergem para uma via final comum que resulta no TPP sendo os principais

mecanismos envolvidos: (1) ativação precoce do eixo hipotálamo-hipófise-adrenal nos compartimentos materno ou fetal, (2) hemorragia decidual, (3) distensão uterina patológica e (4) amplificada resposta inflamatória frente à infecção. Todos esses processos tem em comum a possibilidade de ativação dos mecanismos que levam à dinâmica uterina precoce, o encurtamento do colo do útero e/ou a rotura das membranas corioamnióticas alterações que, curiosamente, surgem antes mesmo que os primeiros sinais clínicos dessas entidades obstétricas sejam reconhecidas (Moroz et al., 2014).

1.2. Mecanismos fisiopatológicos propostos para o determinismo do Trabalho de Parto Pré-Termo

O mecanismo pelo qual o eixo hipotálamo-hipófise-adrenal é prematuramente sensibilizado para atuar como desencadeador do TPP está vinculado às respostas fisiológicas que partem do compartimento materno e/ou fetal.

Estudos observacionais correlacionam o estresse físico ou mental maternos, incluindo entre estes, a ansiedade e a depressão, como de maior risco ao parto pré-termo (Berkowitz et al.,1983; Ding et al., 2014). Outro estudo nesse sentido e de base populacional também evidenciou maior chance de parto pré-termo entre mulheres vítimas de estresse pós-traumático (Lipkind et al., 2010). Admite-se que a ativação do eixo hipotálamo-hipófise-adrenal desencadeie uma cascata de reações neuroendócrinas envolvendo o aumento e liberação do hormônio corticotrófico placentário (McLean et al.,1995; Korebrits et al.,1998), como também, a liberação do hormônio adrenocorticotrófico pela hipófise fetal que, atuando ambos na placenta, elevam as concentrações de estrogênios placentários e prostaglandinas, ativadores primordiais das contrações miometriais que precipitam o início do trabalho de parto (Challis et al., 1989).

A associação de hemorragia de origem genital e elevado risco de TPP e RPM-PT já está bem documentada (Harger et al.,1990, Salafia et al.,1995). O sangramento imotivado procedente da decídua (descolamento da placenta) é consequência de vasos sanguíneos

danificados e apresenta-se clinicamente como hemorragia vaginal ou hematoma retroplacentário configurando um dado preditivo de risco que pode ser observado desde o primeiro trimestre gestacional (Buhimschi et al., 2010).

A elevada concentração do fator de coagulação tecidual, presente no hematoma retrodecidual, é responsánel pela ativação de uma sequência de vias metabólicas da coagulação que resultam na formação de trombina. A trombina, por sua vez, para além das suas propriedades hemostáticas, liga-se a receptores de proteases que regulam a expressão de enzimas, tais como as metaloproteinases, que degradam o colágeno das membranas e favorecem a RPM-PT e consequente nascimento pré-termo (Rosen et al., 2002). Da mesma forma, atribui-se à trombina sua interação com a inibição dos receptores de progesterona fazendo diminuir sua produção e sensibilidade pelas células deciduais e assim permitir a instalação do TPP em função do desequilíbrio hormonal instalado (Goldenberg et al., 2008; Lockwood et al., 2012; Norwitz et al., 2007).

A gestação múltipla, o polidrâmnio e outras formas de distensão uterina excessiva também são fatores de risco bem reconhecidos para o TPP. A sobredistensão miometrial induz à formação de junções do tipo *gap*, à super expressão de receptores de ocitocina, à maior produção de prostaglandina E2 e F2 e à quebra da cadeia leve de miosina. Estes fenômenos representam eventos críticos para o início das contrações uterinas e dilatação cervical (Ou et al.,1997; Word et al.,1993). Da mesma forma, a distensão miometrial também está relacionada ao aumento da expressão de genes que resultam na ativação das vias inflamatórias e da colagenólise (Sooranna et al., 2005), assim como, a própria distensão volumétrica fetal pode contribuir para a sensibilização do miométrio através da liberação de citocinas, prostaglandinas e colagenases produzidas a partir do excessivo estiramento das próprias membranas fetais (Maradny et al.,1996; Nemeth et al., 2000).

A participação do mecanismo inflamatório na etiopatogenia do parto pré-termo, apesar de fortes evidências, ainda se constitui em matéria constante de investigação, uma vez que, as respostas inflamatórias envolvem múltiplas vias bioquímicas complexas e multifacetadas. Nesse contexto geral têm-se postulado que as lesões celulares provocadas

pelos mecanismos inflamatórios, oxidativos e apoptose, isoladamente ou interrelacionados, apresentam-se como bons argumentos para a base biomolecular fisiopatológica da prematuridade.

1.3. Aspectos gerais acerca da anatomofisiologia das membranas corioamnióticas e mecanismo inflamatório dos tecidos gestacionais

As membranas corioamnióticas consistem do âmnion e córion interligados pela matriz extracelular (MEC). Trata-se de um tecido de grande resistência, embora flexível, capaz de suportar e adaptar-se ao crescimento progressivo do volume fetal, líquido amniótico, pressão intrauterina e intra-abdominal. Sua integridade é fundamental para a evolução bem-sucedida da gestação e, principalmente, o bem-estar fetal (Hay, 1981).

A composição histológica das membranas corioamnióticas foi descrita como um tecido constituído por cinco camadas (Figura 1). A camada mais interna, o *âmnio*, é banhada pelo líquido amniótico e formada por uma única placa epitelial de células cúbicas derivadas do ectoderma embrionário. Esse epitélio acha-se firmemente conectado à sua *membrana basal* que o separa de uma outra camada, acelular, composta por *colágenos instersticiais* seguida por uma fileira de células mesenquimais, provavelmente fibroblastos derivados do mesoderma do disco embrionário. A camada mais externa do âmnio é uma *zona esponjosa*, também desprovida de células, contígua à segunda membrana fetal que se designa de *córion* liso. (Cunningham et al.,1997).

A capacidade de tensão e flexibilidade das membranas é fornecida pela riqueza de colágenos do tipo I e III, outros tipos menos frequentes, V, VI e VII, além de componentes não-colagenosos da MEC que inclui a elastina, laminina, proteoglicanos, fibronectina, plasminogênio e integrinas (Malak et al.,1993; Bryant-Greenwood, 1998).

O líquido amniótico, contido no interior da cavidade amniótica, tem sua produção desde as primeiras semanas de gestação até por volta da 20ª semana e origina-se, nesse momento, da passagem passiva de um ultrafiltrado do plasma materno (Brace, 1997). A partir de então a diurese e a degultição fetal representam os mecanismos mais importantes

para sua dinâmica (Wladimiroff et al., 1974) admitindo-se que cerca de 95% do total de líquido amniótico é renovado por dia, notadamente em gestações mais próximas do termo. Sua composição é essencialmente de água (98 – 99%), contendo de 1 a 2% de elementos sólidos representados por substâncias orgânicas e inorgânicas (Brace et al, 1989).

Em um análise mais profunda admite-se que as membranas corioamnióticas não representam apenas uma barreira mecânica para a contenção do liquido amniótico, como se imaginava, mas um componente importante contra a invasão de microrganismos considerando sua ativa participação como elemento de ativação local do sistema imune inato (Romero et al.,2006).

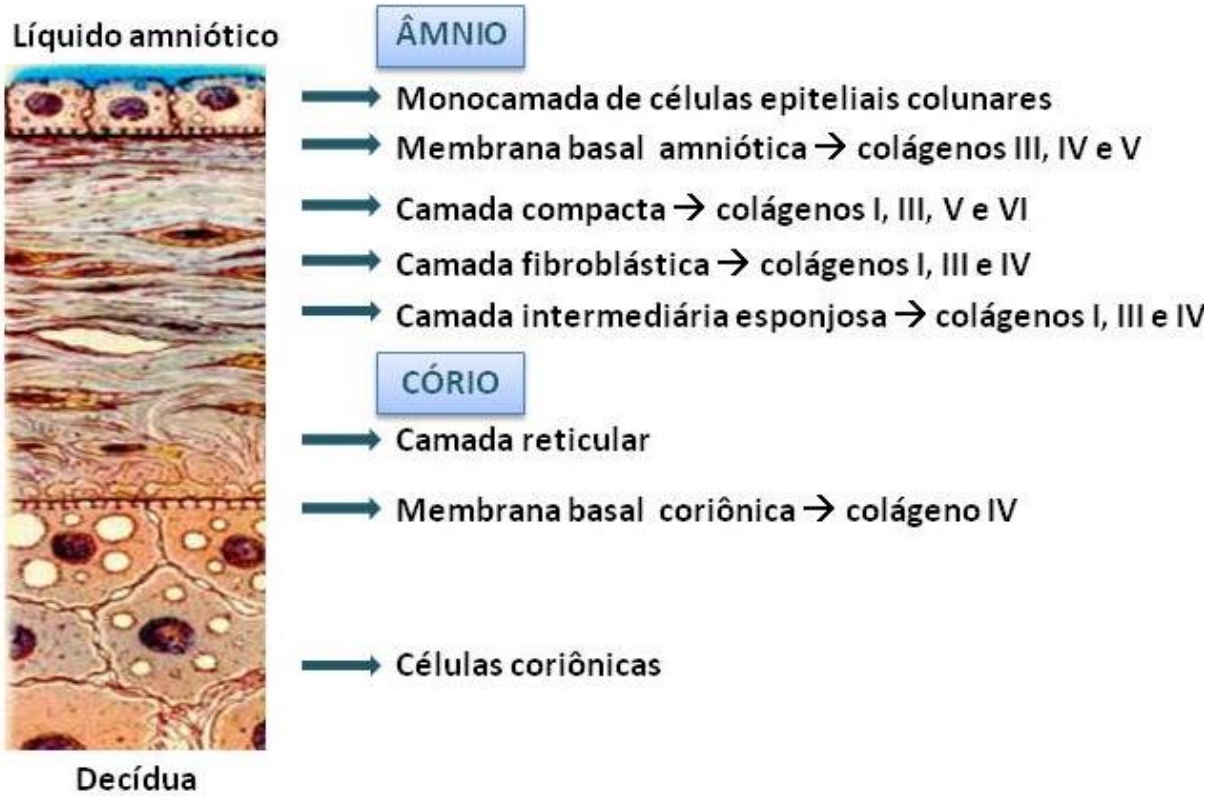


Figura 1. Representação esquemática dos estratos histológicos das membranas corioamnióticas (Parry & Strauss, 1998).

O termo corioamnionite (CAM) ou infecção intra-amniótica (IIA) designa, portanto, o estado no qual é evidenciado um processo inflamatório nos tecidos gestacionais caracterizado pelo comprometimento das membranas corioamnióticas, líquido amniótico, placenta e/ou decídua materna (Arayici et al., 2014). O envolvimento inflamatório do cordão umbilical (artéria, veias umbilicais e a geléia de Wharton) expande o termo para a condição da síndrome de resposta inflamatória fetal (Pacora et al., 2002). Essa definição, em especial a primeira, é, na revisão da literatura, inconsistente, não só para os conceitos clínicos como também para os princípios epidemiológicos, uma vez que, não contribui para o entendimento claro das relações entre a CAM e os desfechos obstétricos (Ericson et al., 2015).

A mais rigorosa definição de CAM é a de uma inflamação nas placas coriônicas e amnióticas das membranas confirmadas através do estudo histopatológico das mesmas o que requer a presença de um infiltrado neutrofílico, ou seja, o limite de pelo menos 10 neutrófilos por campo de grande aumento em uma análise de, ao menos, 10 campos (Hillier et al.,1988) (Figura 2). Entretanto, com o propósito de padronizar os critérios diagnósticos, Redline et al. (2003), classificaram as lesões agudas da placenta em duas categorias: aquelas relacionadas à resposta inflamatória materna e aquelas relacionadas ao feto, assim como, procuraram, também, definir os padrões baseados no estadio e grau do processo inflamatório (Redline et al., 2003).

Segundo esses autores, o termo estadio refere-se à progressão do processo inflamatório agudo dos tecidos afetados e o grau procura definir a intensidade dos danos nessa região anatômica. Para os mesmos, no contexto da resposta inflamatória materna, o estadio 1, **subcorionite aguda e/ou corionite aguda precoce**, caracteriza-se pela presença de neutrófilos nos espaços subcoriônico e/ou coriônico; estadio 2, **corioamninite aguda**, pela inflitração dos neutrófilos no tecido conectivo coriônico e/ou placa coriônica e o estadio 3, **corioamnionite necrotizante**, caracterizado pela necrose que atinge o âmnio. Quanto ao grau, estabelece como **leve a moderado** aquele grupamento de neutrófilos que

difusamente se encontram na placa coriônica, córion ou âmnio, enquanto o grau 2, **severo**, refere-se à presença de pelo menos três microabscessos (Redline et al.,2003).

Esses aspectos permitiram observar que os critérios de estadios parecem ter maior valor clínico que as definições de graduação pois foram mais reprodutíveis entre os observadores no sentido de se estabelecer com mais precisão a severidade do processo inflamatório. A esse respeito, Park et al. (2009), relatam, também, que os critérios de localização do infiltrado neutrofílico nas camadas das membranas ovulares estão muito mais relacionados à resposta inflamatória quando associados às concentrações de citocinas encontradas no líquido amniótico (Park et al.,2009).

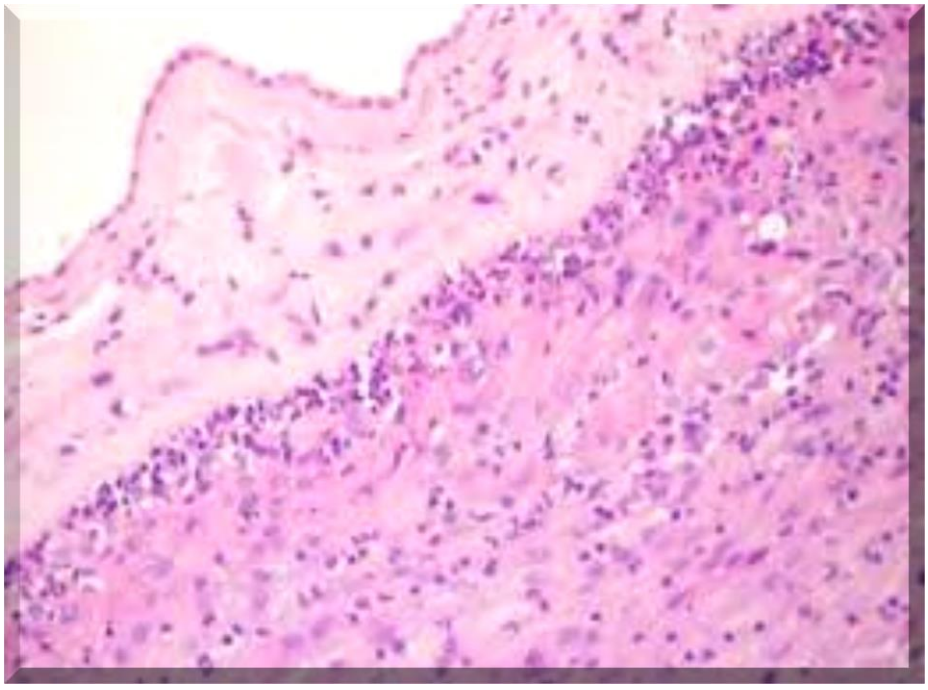


Figura 2. Fotomocrografia das membranas corioamnióticas demonstrando corioamnionite histológica. Evidencia-se epitélio amniótico em monocamada sobre o tecido conjuntivo frouxo avascular e rico infiltrado polimorfonuclear no âmnio e cório. HE. Aumento de 400x.

O mecanismo inflamatório observado no ambiente uterino deve ser compreendido como uma síndrome e, curiosamente, ser interpretado com cautela. O mesmo pode representar um nítido processo de invasão microbiológica, mais comumente compreendido, ou ocorrer em um ambiente de caráter estéril e inflamatório, nesse último caso, com a

ausência de microrganismos demonstráveis e possivelmente induzido por “sinais de perigo” liberados em várias condições fisiopatológicas em que se observa estresse, injúria e/ou morte celular (Romero et al., 2014; Romero et al., 2015).

Os chamados “sinais de perigo”, estudados em pesquisas recentes e que procuram encontrar o elo definitivo envolvendo a etiopatogenia da prematuridade sugerem que os distúrbios maternos da imunidade, inata ou adquirida, estão entre as principais causas de parto pré-termo e englobariam duas condições: aquelas de origem infecciosa e outra ainda não totalmente esclarecida e sem o isolamento específico de agente biológico cujo componente inflamatório provavelmente provém de respostas individuais (Romero et al., 2014).

A esse respeito, Menon (2014), refere-se à intrigante relação entre os processos inflamatórios e os desfechos obstétricos, quer fisiológicos, quer patológicos. Para o autor o trabalho de parto a termo ou mesmo pré-termo tem o envolvimento de mediadores inflamatórios, indicando que, no termo, a manifestação imune-inflamatória parece ser mais restrita e de caráter fisiológico, enquanto que, no nascimento pré-termo, os mecanismos são desencadeados por uma maior atividade inflamatória e de vulto patológico. Essa condição de hiperestimulação inflamatória, observada no parto pré-termo, parece mais relacionada à presença de um estímulo infeccioso como relatado em cerca de 50% dos casos de TPP e, em até, 70% dos casos de RPM-PT (Romero et al., 2006).

Assim, considerando a participação dos agentes biológicos na etiopatogenia da prematuridade admite-se, de forma geral, que a rota da infecção para a cavidade amniótica se dá pela ascensão de bactérias presentes no trato genital inferior, uma vez que, esse sítio anatômico alberga os microrganismos mais envolvidos (Romero et al., 2006). Todavia, apesar de ser a via mais frequente de infecção da cavidade uterina, observa-se que nem todas as gestantes desenvolvem de forma documental a infecção intra-amniótica e essa possível proteção é provavelmente garantida pela presença do muco cervical que se constitui em uma barreira à ascenção dos agentes biológicos durante a gravidez (Hein et al., 2001; Hansen et al., 2014).

Outro dado relevante quanto à etiopatogenia da corioamnionite foi a documentação de que não há, obrigatoriamente, a necessidade da rotura das membranas corioamnióticas para se instalar a infecção intra-amniótica (Galask et al., 1984). A corioamnionite tem sido identificada em casos de trabalho de parto prematuro com membranas íntegras (Combs et al., 2015), em mulheres com insuficiência istmocervical (Oh et al., 2010), em gestantes portadoras de colo uterino encurtado (Romero et al., 2014; Vaisbuch et al., 2010), em casos de hemorragia genital idiopática da gravidez (Gomez et al., 2005) e na placenta prévia (Madan et al., 2010).

Esses exemplos demonstram de maneira muito particular que os agentes biológicos, em especial as bactérias, podem transpor as membranas corioamnióticas e atingir a cavidade uterina a despeito de sua integridade e, em geral, permitir que se desenvolva nos tecidos gestacionais um ambiente de infecção de forma subclínica (Galask et al., 1984; Gravett et al., 1986; Romero et al., 2001).

Outras possíveis rotas de infecção menos frequentes incluem a via hematogênica ou transplacentária. Essas são decorrentes de infecções maternas sanguíneas que se estendem até os espaços intervilosos da circulação fetal como observado, por exemplo, nos casos das doenças periodontais subclínicas e suas íntimas relações com a prematuridade (Offenbacher et al., 2001; Leon et al., 2007). Outras vias como a infecção retrógrada da pelve e a infecção transuterina, esta última, causada por procedimentos como a amniocentese, são eventos de muito maior raridade (Fahey, 2008).

Os mais frequentes microrganismos encontrados na cavidade amniótica de gestantes com infecção intra-amniótica são *Ureaplasma urealitycum* e *Mycoplasma hominis* e espécies de *Fusobacterium*; ocasionalmente também são encontrados bacilos Gram-negativos, como os do gênero *Bacteroides* e *Prevotela*, ambos presentes na vaginose bacteriana; coliformes como *Escherichia coli* e *Streptococcus* do grupo B (Sperling et al.,1988; Romero et al.,1994; Gibbs et al.,1982), com destaque, nesse cenário, para o *core* patológico da vaginose bacteriana os quais se instalam nas membranas e invadem a cavidade amniótica proliferando-se no líquido amniótico (Kim et al., 2010). Admite-se, ainda,

que o desequilíbrio da microbiota vaginal, representado pela vaginose bacteriana, possa contribuir para o gatilho da resposta inflamatória caracterizada por maior concentração de interleucinas (IL) -1β, IL-6 e IL-8 e de sialidades que podem representar um fator de maior risco para resultados gestacionais adversos (Ferreira et al., 2015; Discacciati et al., 2011).

A infecção intra-amniótica ou corioamnionite, como já referido, pode, ainda, se apresentar nas condições clínica e histológica. A corioamnionite clínica acomete cerca de 1% a 2% dos partos de termo e 5% a 10% dos partos pré-termo, havendo aumento da sua incidência quando há RPM-PT por tempo prolongado (Gibbs et al.,1991). Seu diagnóstico é geralmente definido pela presença de temperatura ≥ 37,8°C e pelo menos dois dos seguintes achados: taquicardia materna (acima de 120 bpm), leucocitose materna (acima de 18.000 células/mm^3), presença de sensibilidade uterina, líquido amniótico purulento ou de odor fétido ou taquicardia fetal (acima de 160 bpm) (Ramsey et al., 2005). A forma histológica de corioamnionite tem sido descrita em aproximadamente 20% das gestações de termo e em mais de 50% dos partos pré-termo, sendo que a maioria dos casos não são seguidos de sinais clínicos ou sintomas de infecção (Edwards, 2005; Conti et al., 2015; Newton, 2005; Menon et al., 2010).

Considerando os agentes biológicos como fatores fundamentais na etiopatogenia do parto pré-termo há, nesse sentido, clareza que a resposta inflamatória subsequente é protagonizada pela participação de *Toll-Like Receptors* (TLRs) que merecem destaque devido seu papel crucial contra a invasão microbiana tendo em vista sua capacidade de reconhecer amplo espectro de padrões moleculares (Brodsky et al., 2007; DiGiulio et al., 2008).

O reconhecimento dos Padrões Moleculares Associados à Patógenos ($PAMP_s$) pelos TLRs ativa diversas vias de sinalização intracelulares que culminam com a ativação de fatores de transcrição responsáveis pela expressão de genes envolvidos nas respostas inflamatórias e antivirais (Anwar et al., 2013; Kawaiet al., 2010). Todas essas vias são iniciadas pela interação do TLR com seu ligante específico na superfície da célula ou nos compartimentos celulares o que leva a dimerização dos mesmos. Essa dimerização resulta

na aproximação dos domínios do Receptor Toll / IL-1 (TIR) de um TLR com o domínio TIR do outro. Em seguida são recrutadas moléculas adaptadoras que participam do quimiotaxia de outras proteínas e da ativação de diversas quinases (Abbas et al., 2012). Dentre os fatores de transcrição ativados pelas vias de sinalização dos TLRs destaca-se o Fator Nuclear κB (NF-κB), a proteína ativadora 1 (AP-1), o Fator Regulador de Interferon 3 (IRF3) e o Fator Regulador de Interferon 7 (IRF7) (Abbas et al., 2012). A ativação de NF-κB e AP-1 resulta na produção de citocinas pró-inflamatórias, quimiocinas e moléculas de adesão endotelial, enquanto que a ativação de IRF3 e IRF7 resulta na produção de interferons (IFN) do tipo I (IFN-α e IFN-β), os quais atuam nas respostas anti-virais (Abbas et al., 2012; Murphy et al., 2010).

Essa resposta inflamatória mediada pelos TLR_s é, em última análise, motivada pelo recrutamento de neutrófilos ativados e, em menor grau, por macrófagos e mediadores pró-inflamatórios. Esses mediadores inflamatórios são os resposáveis pela indução da ciclooxigenase-2 (COX-2) nas células do âmnio e decídua, assim como, pela produção de prostaglandinas e metalopreoteinases que deflagram as contrações uterinas e fragilizam as membranas corioamnióticas até a sua rotura, respectivamente (Challis et al.,2001).

Entre os diversos mecanismos endógenos de deflagração do nascimento pré-termo, e, em associação aos fatores de risco acima mencionados, vale enfatizar o papel do estresse oxidativo como, também, uma via etiopatogênica envolvida e intrinsicamente relacionada à atividade inflamatória e os resultados gestacionais adversos, notadamente aqueles que se relacionam à prematuridade.

1.4. A formação de radicais livres e o estado de estresse oxidativo

O fornecimento de energia para a célula provém de um complexo mecanismo respiratório com sede na mitocôndria. Durante esse processo são liberados pelo sistema de transferência de elétrons formas reativas de oxigênio que permanecem disponíveis como metabólitos que participam dos mecanismos homeostáticos celulares (Droge, 2002).

As formas reativas de oxigênio, também denominadas de espécies reativas de oxigênio (ERO_s), são radicais livres (RL_s) que possuem um único elétron sem um par correspondente localizado na órbita externa do átomo cuja energia criada é, por essa particular configuração, instável, efêmera e liberada através de reações moleculares subsequentes compartilhadas com outras substâncias químicas, orgânicas e inorgânicas, tais como proteínas, lipídeos – principalmente aquelas moléculas constituintes das membranas celulares – e ácidos nucleicos (Hensley et al., 2000).

As ERO_s são representadas pelo ânion superóxido (O_2^-), molécula derivada do O_2 após reações enzimáticas oxidativas, que ocorre em diversos segmentos celulares como no retículo endoplasmático, mitocôndrias, membrana citoplasmática, peroxissomos e citosol. Uma vez submetido à reação de dismutação pela enzima superóxido dismutase o O_2^- é convertido em peróxido de hidrogênio (H_2O_2) que, na presença de cofatores como o Cu^{2+} / Fe^{2+}, pode ser catalisado para a formação do radical hidroxila (OH^-), um segundo e potente mediador de reações oxidativas (Beckman et al.,1990; Mouithys-Mickalad et al.,1999).

Outra importante fonte de liberação de radicais livres são aqueles derivados do óxido nítrico, também conhecido como fator de relaxamento derivado do endotélio, cuja finalidade é regular o fluxo sanguíneo e inibir a agregação das plaquetas e das células brancas no contato com a parede vascular (Webster et al., 2008). A ação pró-oxidante do óxido nítrico é atribuída a reações intermediárias que envolvem o nitrogênio e que, ao reagir com o O_2^-, formam o peroxinitrito ($ONOO^-$). Ambos possuem um elétron não compartilhado o que os torna um radical livre altamente reativo capaz de danificar macromoléculas e, em conjunto com outros mediadores inflamatórios, lesionar a célula (Vega et al.,1998; Rosselli et al.,1998).

O peroxinitrito é um potente oxidante para a maioria das moléculas e por sua capacidade de rápida difusão transforma-se em outros radicais livres como: radical hidroxil (OH^-) e radical nitro (NO_2), resíduos responsáveis pelos fenômenos de hidroxilação e nitração, respectivamente (Mouithys-Mickalad et al.,1999).

Da mesma forma que EROs, as espécies reativas de nitrogênio (ERNs) têm sido associadas a condições patológicas como aquelas derivadas dos fenômenos de isquemia/reperfusão, choque séptico e aterosclerose (Reynaert et al.,2005; Schrier et al., 2004), bem como aos processos que envolvem o sistema imune envolvendo os monócitos, macrófagos e lipopolissacarídeos tendo como resposta a produção de citocinas pró-inflamatórias (Rosselli et al.,1998; Osborn et al.,2002).

Em contrapartida à produção dos radicais livres, fisiologicamente produzidos pelo organismo humano e, particularmente presentes no período gestacional, as células procuram manter um balanço estável entre os mesmos e os mecanismos antioxidantes (Agarwalet al., 2012) representados em três níveis distintos de atividade: (a) prevenção na formação de RL_s (b) eliminação de RL_s formados e (c) reparo de moléculas danificadas pelas RL_s (Sies, 1993). Nesse contexto e, em condições de homeostase, o compartimento intracelular disponibiliza moléculas com capacidades redutoras ou antioxidantes.

É importante salientar que em concentrações aceitáveis os radicais livres, produzidos nas reações fisiológicas, frequentemente participam dos sistemas biológicos e são importantes para a divisão e sobrevivência celular, e, são, ainda, mediadores moleculares de sinalização para os processos inflamatórios, para ativação da autoimunidade, da autofagia, da apoptose e resposta ao estresse (Sies, 1991).

A mensuração, *in vivo*, dos radicais livres é possível embora esteja sujeita a dificuldades de detecção em função da presença de artefatos metodológicos. Em geral, procura-se analisar aqueles onde, na sua estrutura molecular, acha-se disponível a presença de um elétron livre, altamente reativo, obtendo-se, assim, o intuito de aumentar a sensibilidade do método (Valgimigli et al.,2001).

Entre os biomarcadores do estresse oxidativo, universalmente utilizados, pode-se citar os produtos da peroxidação lipídica como o malondialdeído (MDA), um dialdeído formado a partir da oxidação dos ácidos graxos poliinsaturados que, entre eles, destaca-se, o ácido araquidônico cujo subproduto é detectado como um dos reagentes do ácido

tiobarbitúrico (TBAR) (Lima et al., 2001). Pode-se, ainda, pesquisar o biomarcador 3-nitrotirosina (3-NT), um subproduto do dano oxidativo ocasionado pela nitração das proteínas a partir do peroxinitrito (Myatt, 2010) e, por fim, a detecção do dano oxidativo ao ácido desoxirribonucléico (DNA) que pode ser revelado por alterações na estrutura de suas bases nitrogenadas, em especial a guanina, pela medida dos níveis de 8-hidroxi-2'-deoxiguanosina (8-OHdG) (Menon, 2014).

Vale ressaltar que estes marcadores não são específicos para a atividade de uma ERO_s ou ERN_s em particular, assim como, não o são para o tecido no qual presume-se que esteja ocorrendo o estresse oxidativo. Assim sendo, a mensuração de múltiplos marcadores torna ainda mais específica a presença da lesão oxidativa e, idealmente, devem ser analisados no tecido ou líquido supostamente submetido às agressões bioquímicas (Henrotin et al.,2009).

1.5. O estresse oxidativo e sua participação no trabalho de prto prematuro e na rotura prematura das membranas pré-termo

Estudos recentes têm atribuído ao estresse oxidativo uma participação importante nos processos relacionados à reprodução, quer fisiológicos, quer patológicos, como por exemplo, sua interação no fenômeno de deflagração do trabalho de parto por ocasião do termo da gestação, ou, sua participação nos mecanismos etiopatogênicos da pré-eclâmpsia, aborto de repetição e prematuridade (Agarwal et al.,2005; Hung et al.,2010; Chai et al., 2012). No campo da prematuridade, os estudos relacionam o estresse oxidativo aos mecanismos inflamatórios como componentes inseparáveis e, embora, presentes em patologias distintas eles parecem ter papéis importantes como vias efetoras que convergem para a deflagração do nascimento pré-termo (Menon, 2014).

Nesse sentido, a gestação, pelas suas peculiaridades, transforma-se em um ambiente propício ao desequilíbrio entre a produção e controle dos radicais livres. Os tecidos gestacionais, uma vez submetidos a uma maior concentração de RL_s, podem sofrer

as consequências do dano oxidativo que, em conjunto com a exacerbação da atividade inflamatória, dá origem a um ciclo vicioso com características potencialmente patológicas. A possibilidade de dano celular oxidativo se manifesta em todos os componentes celulares, porém o DNA é um dos alvos críticos para onde se dirigem os $RL_{s.}$ Essa lesão se dá em consequência da íntima relação deles com cadeia respiratória mitocondrial e, em grande parte, ao bloqueio da enzima aconitase, proteína mitocondrial que participa das reações entre o Fe^{2+} e H_2O_2, moléculas fundamental para a ativação das reações de Fenton e Haber-Weiss, suscitando, assim, a formação de radicais hidroxila que, como um metabólito, são capazes de fomentar a nitração das proteínas mitocondriais, gerar a oxidação do DNA nuclear, desencadear alterações estruturais no telômero e amplificar a produção de O_2^- mantendo, assim, a continuidade do estresse oxidativo (Orrenius et al.,2011).

O dano ao DNA se caracteriza, molecularmente, por quebras em suas cadeias simples ou duplas, trocas entre cromátides irmãs, reticulação do DNA ou mesmo modificações nas bases nitrogenadas (Richteret al., 1988; Fraga et al.,1990). Entre estas, a base mais suscetível a alterações induzidas pelos $RL_{s,}$ é a guanina, cujo produto de oxidação é representado pelo metabólito 8-OHdG que, em elevadas concentrações, indica claramente um processo patológico (Subash et al.,2010).

De forma semelhante os RL_s podem ter como alvo as membranas fosfolipídicas e nelas determinar importantes modificações estruturais. Para alguns autores, representa o evento citotóxico primário em função da capacidade dos mesmos em reagir com os ácidos graxos poliinsaturados permitindo o processo de peroxidação lipídica representando um fator desencadeador da falência dos mecanismos de troca dos metabólitos, do desequilíbrio dos nutrientes celulares e da clivagem das cadeias do próprio DNA que, em situações extremas de grande duração e extensão no processo de oxidação, culminam com a morte celular (Benzie, 1996).

Entretanto, o mecanismo de estresse oxidativo extrapola o nível intracelular e se estende ao meio extracelular proporcionando a desestruturação dos componentes da matriz extracelular dirigindo-se aos proteoglicanos, ao colágeno e à elastina contribuindo, nesse

ambiente, para os fenômenos que culminam com o nascimento pré-termo (Lima et al., 2001).

1.6. Fundamentos básicos dos mecanismos ativadores da apoptose nos tecidos gestacionais

Envelhecimento celular e apoptose, embora com algumas semelhanças, caracterizam-se como fenômenos distintos que se encontram presentes nos processos patológicos da prematuridade. O conceito de senescência é um fenômeno que pressupõe uma associação entre a necessária parada do processo de divisão celular com uma atividade inflamatória subsequente (Menon, 2014). Apoptose, ao contrário, é um fenômeno de morte celular programada em que, pela sua rápida progressão, não há, por parte do hospedeiro, a concomitância de atividade inflamatória (Kumar et al.,2012).

Polettini et al. (2015), referem-se ao tema admitindo que o envelhecimento dos tecidos placentários pode ter um papel importante nos resultados adversos da gestação permanecendo, porém, a dúvida de como interpretá-los no contexto dos mecanismos da prematuridade, se como eventos dependentes ou apenas interrelacionados.

Assim sendo, estudos indicam que o processo de envelhecimento celular pode, principalmente em condições de estresse oxidativo crônico, desencadear efeitos adversos como a incapacidade de regeneração tecidual e a liberação de moléculas bioativas, incluindo, entre estas, radicais livres, citocinas pró-inflamatórias, quimiocinas, eicosanóides, metaloproteinases e fatores como os de crescimento e angiogênicos. Esses elementos em conjunto compreendem o que atualmente se denomina como fenótipo secretor associado à senescência (Correia-Melo et al.,2014).

Na tentativa de elucidar os mecanismos que envolvem esse fenômeno foi possível determinar, *in vitro*, que células expostas a um fator reconhecidamente relacionado ao estresse oxidativo, como os resíduos do fumo, podem provocar dano oxidadivo celular, em especial ao DNA, e interferir na fisiologia dos telômeros proporcionando seu encurtamento e consequentemente um fenótipo de senescência (Menon, 2014; Menon et al., 2012). O

encurtamento observado nos telômeros é consequência da perda dos fatores protetores do cromossomo (de Lange, 2002) e/ou motivado por danos focais ao DNA, em geral, relacionados à persistência do agente indutor (Correia-Melo et al., 2014). Outro aspecto curioso das pesquisas relacionadas ao envelhecimento celular é que, após exposição das células às substâncias derivadas do fumo, houve a ativação das proteinoquinases, especificamente a p38 proteinoquinase ativada por mitógenos (p38 MAPK), que, uma vez fosforilada em seus receptores específicos, promove também o bloqueio do ciclo celular (Brancho et al., 2003).

Neste contexto, baseado nas pesquisas que reconheceram as vias metabólicas da senescência celular, os autores propõem que a presença do processo inflamatório, observado nas condições de prematuridade, não é exclusivamente fruto da migração de fagócitos mas decorrentes do estresse oxidativo como um desencadeador do processo de envelhecimento placentário relacionado à ativação do chamado fenótipo secretor associado à senescência cujas manifestações se traduzem pela marcante ação inflamatória em concomitância com metabólitos procedentes do próprio dano oxidativo.

Em outro contexto, pesquisas têm procurado definir o papel da apoptose no mecanismo etiopatogênico da prematuridade. Múltiplas vias estão sendo estudadas nesse sentido e, isoladamente ou em sinergismo, parecem confluir para o desfecho da morte celular. O que há de comum entre elas é a participação da proteína p53. Molécula codificada por um gene situado no cromossomo de número 17, o qual leva o mesmo nome (gene *p53*) em consequência de seu peso molecular de 53 kDa, cuja principal função está relacionada à preservação da integridade do código genético em cada célula, ou seja, à manutenção da mesma sequência de nucleotídeos ao longo de toda a molécula de DNA igualmente presente em cada célula do organismo humano (Pinho, 2000; Irwin et al., 2001).

Durante o ciclo de divisão celular a proteína p53 é responsável por uma verificação quanto à eventual ocorrência de mutação na sequência do código genético. Caso seja verificada a existência de mutação, é função da proteina p53, o desdobramento de uma cascata de reações com o intuito de impedir que essa célula entre em processo de mitose e

complete a divisão celular (Vousden et al.,2002). Na vigência de um dano recorrente ao DNA, processos inflamatórios ou mesmo fatores ainda desconhecidos (Fortunato et al., 2001), múltiplas vias relacionadas à apoptose são ativadas e entre elas citam-se: (1) aquelas relacionadas ao mecanismo de reparo do DNA, (2) aquelas decorrentes da fragmentação do DNA, (3) aquelas subsequente à ativação do complexo entre o fator de necrose tumoral-α e o seu receptor ligante Fas (TNF-α – FasL), (4) aquela procedente de uma maior expressão de metaloproteinases ou bloqueio do fator tecidual de inibição de metaloproteinaes.

Uma dessas vias reporta-se ao contínuo mecanismo de reparo do DNA, notadamente à base nucleotídica guanina, subsequente à agressão gerada pelos radicais livres. O estresse oxidativo, não controlado pelos mecanismos antioxidantes, pode desencadear reações moleculares como determinar a conjugação das proteínas 8-Oxoguanina glicosilase (OGG1) ao radical livre 8-OHdG formando o complexo enzimático OGG1:8-OHdG. Esse complexo tem por característica ativar a enzima trifosfato guanosina hidroxilase (Ras-GTPase) que estimula a transcrição da proteína p53 resultando na apoptose das células coroamnióticas como descrito por Menon (2014) e Klungand et al (2017) e tendo como desfecho subsequente RPM-PT (Menon, 2014; Klungand et al., 2007).

Dando continuidade aos eventos que se seguem à incapacidade de reparo do DNA e à sua fragmentação ocorre a ativação da proteína p53 responsável pela expressão das proteinas *bax* e *Bcl-2*. Essas proteinas em condições de homeostase acham-se em equilíbrio numa proporção de 1:1 e são responsáveis, respectivamente, em promover a apoptose e a proliferação celular (Reed, 1994).

Na condição de superexpressão da proteina *bax*, pela maior concentração de p53, dá-se início aos mecanismos de desestruturação da membrana mitocondrial e posterior liberação do citocromo C permitindo, assim, a conversão da pró-caspase 9 para sua forma ativa e subsequente cascata de caspases efetoras (caspases 2, 3, 6 e 7), também relacionadas à apoptose. Uma maior expressão da proteína p53 pode, ainda, inibir a produção de *Bcl-2*, um agente anti-apoptótico, resposável pela integridade da membrana

mitocondrial e contenção do citocromo C responsável pela cascata de reações bioquímicas descritas anteriormente (Menon et al., 2007).

Outras vias podem, ainda, ser mencionadas e também determinam a apoptose como aquelas que envolvem a interação entre o fator de necrose tumoral-α e o seu receptor ligante Fas (TNF-α – FasL). Essa via dá início a sinais de transdução celular com a formação de outros complexos proteicos como: (a) receptor TNF-α associado ao domínio de morte (TRADD) e (b) Fas associado ao domínio de morte (FADD). Esses complexos se caracterizam por também ativarem as caspases iniciadoras e efetoras da apoptose (Menon et al.,2002; Menon et al., 2007).

A maior concentração da proteina p53 pode, ainda, estar relacionada ao mecanismo que envolve a superexpressão gênica de metaloproteinases, em especial a MMP-2, assim como, o bloqueio do fator tecidual inibidor de metaloproteinases (TIMP). Essa combinação de eventos culmina com a modificação estrutural dos tecidos gestacionais e a deflagração do parto no termo, em especial, na RPM-PT e no TPP (Fortunato et al.,2000).

Busca-se, nessa revisão de literatura, compreender os múltiplos mecanismos envolvidos na etiopatogenia e fisiopatologia da prematuridade admitindo que esses fenômenos fisiológicos, permanecendo sob controle homeostático, participam do curso bem-sucedido da gestação e que uma possível interferência de fatores intrínsecos ou extrínsecos ao ambiente humano parecem transformá-los em gatilhos importantes para o desequilíbrio dos mesmos e permitir suas participações em resultados gestacionais adversos.

Justificativa

2. Considerando que o parto pré-termo:

1. Apresenta-se como um problema médico, social e humano que tem elevada taxa de incidência global oscilando entre 5 – 18% e com prevalência estável ao longo de décadas;

2. Que seu entendimento etiopatogênico e fisiopatológico ainda não se encontra totalmente esclarecido e que múltiplos mecanismos estão implicados na sua gênese;

3. Que, entre esses mecanismos, os processos inflamatórios dos tecidos gestacionais estão possivelmente implicados nos resultados perinatais adversos;

4. Que outros mecanismos como o estresse oxidativo e a apoptose aparecem como possíveis fatores interrelacionados;

Propusemos-nos a avaliar mecanismos fisiopatológicos relacionados ao parto pré-termo, incluindo a análise do dano de DNA em membranas corioamnióticas de gestações complicadas por prematuridade, a relação existente entre o dano ao DNA, a corioamnionite histológica e a prevalência da apoptose e finalmente a análise da associação de resultados adversos neonatais e corioamnionite histológica na vigência de prematuridade.

Objetivos

3. Objetivo Geral

O objetivo desse estudo é analisar os mecanismos fisiopatológicos relacionados ao parto pré-termo espontâneo: corioamnionite histológica, estresse oxidativo, apoptose e a associação entre a corioamnionite histológica e resultados neonatais adversos.

3.1. Objetivos específicos

3.1.1. Avaliar os níveis de 8-oxo-2'-deoxiguanosina (8-OHdG) em membranas corioamnióticas de gestações à termo e naquelas complicadas pelo nascimento pré-termo espontâneo incluindo, entre estes, aqueles vinculados ao Trabalho de Parto Prematuro com membranas íntegras e a Rotura Prematura de Membranas Pré-Termo;

3.1.2. Analisar a ocorrência da expressão da proteína p53 em membranas corioamnióticas de gestações pré-termo e sua associação com os níveis de 8-OHdG e a presença de corioamnionite histológica;

3.1.3. Analisar os resultados neonatais adversos em gestações pré-termo associados à corioamnionite histológica.

Referências Bibliográficas

Abbas AK, Lichtman AH. Imunologia celular e molecular. 7ª Edição. [Rio de Janeiro]: Elsevier; 2012.

Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol.2012; 10:10-49.

Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 2005;3:1-21.

Ananth CV, Vintzileos AM. Epidemiology of preterm birth and its clinical subtypes. J Matern Fetal Neonatal Med. 2006; 19: 773–782.

Anwar MA, Basith S, Choi S. Negative regulatory approaches to the attenuation of Toll-like receptor signaling. Exp Mol Med. 2013;45: e11.

Avchen RN, Scott KG, Mason CA. Birth weight and school-age disabilities: a population-based study. Am J Epidemiol. 2001; 154: 895–901.

Arayici S, Kadioglu Simsek G, Oncel MY, Eras Z, Canpolat FE, Oguz SS, Uras N, Zergeroglu S, Dilmen U. The effect of histological chorioamnionitis on the short-term outcome of preterm infants <32 weeks: a single-center study. J Matern Fetal Neonatal Med. 2014:27:1129-1133.

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA.1990;87:1620-1624.

Benzie IF. Lipid peroxidation: a review of causes, consequences, measurements and dietary influences. Int J Food Sci. 1996:47:233-261.

Berkowitz GS, Kasl SV. The role of psychosocial factors in spontaneous preterm delivery. J Psychosom Res.1983; 27:283-290.

Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera Garcia, Rohde S, Say L, Lawn JE. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications.Lancet. 2012; 379: 2162-2172.

Brace RA. Amniotic fluid volume regulation. Clin Obstet Gynecol. 1997;40:280-289.

Brace RA, Wolf EJ. Normal amniotic fluid volume changes throughout pregnancy. Am J Obstet Gynecol. 1989; 161:382-388.

Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA, Davis RJ.Mechanism of p38 MAP kinase activation in vivo. Genes Dev. 2003; 17:1969-1978.

Brodsky I, Medzhitov R. Two modes of ligand recognition by TLRs. Cell. 2007; 130:979-981.

Bryant-Greenwood GD. The extracellular matrix of the human fetal membranes: structure and function. Placenta.1998;19:1-11.

Buhimschi CS, Schatz F, Krikun G, Buhimschi LA, Lockwood CJ.Novel insights into molecular mechanisms of abruption-induced preterm birth. Expert Rev Mol Med. 2010; 12:e35.

Chai M, Barker G, Menon R, Lappas M. Increased oxidative stress in human fetal membranes overlying the cervix from term non-labouring and post labour deliveries. Placenta. 2012; 33: 604-610.

Challis JR, Hooper S. Birth: outcome of a positive cascade. Baillieres Clin Endocrinol Metab. 1989; 3:781-793.

Challis JR, Lye SJ, Gibb W, Whittle W, Patel F, Alfaidy N. Understanding preterm labor. Ann N Y Acad Sci 2001; 943: 225-234.

Combs CA, Garite TJ, Lapidus JA, Lapointe JP, Gravett M, Rael J, Amon E, Baxter JK, Brady K, Clewell W, Eddleman KA, Fortunato S, Franco A, Haas DM, Heyborne K, Hickok DE, How HY, Luthy D, Miller H, Nageotte M, Pereira L, Porreco R, Robilio PA, Simhan H, Sullivan SA, Trofatter K, Westover T. Detection of microbial invasion of the amniotic cavity by analysis of cervicovaginal proteins in women with preterm labor and intact membranes. Am J Obstet Gynecol.2015; 212:482e1-482e12.

Conti N, Torricelli M, Voltolini C, Vannuccini S, Clifton VL, Bloise E, Petraglia F. Term histologic chorioamnionitis: a heterogeneous condition. Eur J Obstet Gynecol Reprod. 2015; 34-38.

Corrêa MD, Júnior MDC. Parto Pretermo. In: Rezende J. Obstetrícia. 9.ed. Rio de Janeiro: Guanabara Koogan; 2002; p.892-914.

Correia-Melo C, Hewitt G, Passos JF. Telomeres, oxidative stress and inflammatory factors: partners in cellular senescence? Longevity & Healthspan.2014;3:1-9.

Cunninggham FG, MacDonald PC, Gant FN, et al. Williams Obstetrícia. 20ª Edição. [Rio de Janeiro]: Guanabara Koogan; 1997

de Lange T. Protection of mammalian telomeres. Oncogene. 2002;21:532-540.

DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, Kim CJ, Erez O, Edwin S, Relman DA. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. PLoS One. 2008;3:e3056.

Ding XX, Wu YL, Xu SJ, Zhu RP, Jia XM, Zhang SF, Huang K, Zhu P, Hao JH, Tao FB. Maternal anxiety during pregnancy and adverse birth outcomes: a systematic review and meta-analysis of prospective cohort studies. J Affect Disord. 2014; 159:103-110.

Discacciati MG, Simoes JA, Silva MG, Marconi C, Brolazo E, Costa ML, Cecatti JG. Microbiological characteristics and inflammatory cytokines associated with preterm labor. Arch Gynecol Obstet. 2011;283:501-508.

Droge W: Free radicals in the physiological control of cell function. Physiol Rev. 2002;82:47-95.

Edwards RK. Chorioamnionitis and labor. Obstet Gynecol Clin North Am. 2005;32:287-296.

Ericson JE, Laughon MM. Chorioamnionitis implications for the neonate. Clin Perinatol. 2015:42:155-165.

Fahey JO. Clinical management of intra-amniotic infection and chorioamnionitis: a review of the literature. J Midwifery Womens Health. 2008;53:227-235.

Ferreira CST, Marconi C, Parada CM, Duarte MT, Gonçalves AP, Rudge MV, Silva MG. Bacterial vaginosis in pregnant adolescents: proinflammatory cytokine and bacterial sialidase profile. Cross-sectional study.São Paulo Med J. 2015;133:465-470.

Fortunato SJ, Menon R, Bryant C, Lombardi SJ. Programmed cell death (apoptosis) as a possible pathway to metalloproteinase activation and fetal membrane degradation in premature rupture of membranes. Am J Obstet Gynecol. 2000;182:1468-1476.

Fortunato SJ, Menon R. Distinct molecular events suggest different pathways for preterm labor and premature rupture of membranes. Am J Obstet Gynecol. 2001;184:1399-1406.

Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. Proc Natl Acad Sci USA. 1990; 87:4533-4537.

Galask RP, Varner MW, Petzold CR, Wilbur SL. Bacterial attachment to the chorioamniotic membranes. Am J Obstet Gynecol. 1984;148:915-928.

Gibbs RS, Blanco JD, St Clair PJ, Castaneda YS. Quantitative bacteriology of amniotic fluid from women with clinical intraamniotic infection at term. J Infect Dis. 1982;145:1-8.

Gibbs RS, Duff P. Progress in phathogenesis and management of clinical intraamniotic infection. Am J Obstet Gynecol. 1991;164:1317-1326.

Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet.2008;371:75-84.

Gomez R, Romero R, Nien JK, Medina L, Carstens M, Kim YM, Chaiworapongsa T, Espinoza J, González R. Idiophatic vaginal bleeding during pregnancy as the only clinical manifestation of intrauterine infection. J Matern Neonatal Med. 2005;18:31-37.

Gravett MG, Hummel D, Eschenbach DA, Holmes KK. Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. Obstet Gynecol. 1986;67:229-237.

Hamilton BE, Martin JA, Ventura S. Births: Preliminary Data for 2008. Atlanta, Georgia: Centers for Disease Control. US Department of Health and Human Services US Department of Health and Human Services. 2010.

Hansen LK, Becher N, Bastholm S,Glavind J, Ramsing M, Kim CJ, Romero R, Jensen JS, Uldbjerg N. The cervical mucus plug inhibits, but does not block, the passage of ascending bacteria from the vagina during pregnancy. Acta Obstet Gynecol Scand. 2014;93:102-108.

Harger JH, Hsing AW, Tuomala RE, *et al*. Risk factors for preterm premature rupture of fetal membranes: a multicenter case-control study. Am J Obstet Gynecol. 1990;163:130.

Hay ED. Extracellular matrix. J Cell Biol.1981;91:205_s-223_s.

Hein M, Helmig RB, Schonheyder HC, Ganz T, Uldbjerg N. An in vitro study of antibacterial properties of the cervical mucus plug in pregnancy. Am J Obstet Gynecol. 2001;185:586-592.

Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free Radic Biol Med. 2000;28:1456-1462.

Henrotin Y, Deberg M, Mathy-Hartert M, Deby-Dupont G. Biochemical biomarkers of oxidative collagen damage. Adv Clin Chem. 2009;49:31-55.

Hillier SL, Martius J, Krohn M, Kiviat N, Holmes KK, Eschenbach DA. A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. N Eng J Med. 1988;319:972-978.

Hung TH, Lo LM, Chiu TH, Li MJ, Yeh YL, Chen SF, Hsieh TT. A longitudinal study of oxidative stress and antioxidant status in women with uncomplicated pregnancies throughout gestation. Reprod Sci. 2010;17:401-409.

Irwin MS, Kaelin WG. p53 family uptodate: p73 and p63 develop their own identities. Cell Growth Differ. 2001;12:337-349.

Kawai T, Akira S. The role of pattern-recognition receptor in innate immunity: update of Toll-like receptors. Nat Immunol. 2010; 11:373-384.

Korebrits C, Ramirez MM, Watson L, Brinkman E, Bocking AD, Challis JR. Maternal corticotropin-releasing hormone is increased with impending preterm birth. J Clin Endocrinol Metab. 1998;83:1585-1591.

Kumar V, Abbas Ak, Fausto N. Patologia – Bases Patológicas das Doenças. 7ªEdição. [Rio de Janeiro]: Elsevier; 2012.

Klungland A, Bjelland S. Oxidative damage purines in DNA: role of mammalian Ogg1. DNA repair (Amst). 2007;6:481-488.

Leon R, Silva N, Ovalle A, Chaparro A, Ahumada A, Gajardo M, Martinez M, Gamonal J. Detection of Porphyromonas gingivalis in the amniotic fluid in pregnant women with a diagnosis of threatened premature labor. J Periodontol. 2007;78:1249-1255.

Lima ES, Abdalla DSP. Peroxidação lipídica: mecanismo e avaliação em amostras biológicas. Bra J Pharmac Sci. 2001:37:293-303.

Lipkind HS, Curry AE, Huynh M, Thorpe LE, Matte T. Birth outcomes among offspring of women exposed to the September 11, 2001, terrorist attacks. Obstet Gynecol. 2010;116:917-925.

Liu L,Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, Rudan J, Campbell H, Cibulskis R, Li M, Mathers C, Black RE, Child Health Epidemiology Reference Group opf WHO and UNICEF. Lancet. 2012; 379: 2151-2161.

Lockwood CJ, Kayisli UA, Stocco C, Murk W, Vatandaslar E, Buchwalder LF, Schatz F. Abruption-induced preterm delivery is associated with thrombin-mediated functional progesterone withdrawal in decidual cells. Am J Pathol. 2012; 181:2138-2148.

Madan I, Romero R, Kusanovic JP, Mittal P, Chaiworapongsa T, Dong Z, Mazaki-Tovi S, Vaisbuch E, Alpay Savasan Z, Yeo L, Kim CJ, Hassan SS. The frequency and clinical

significance of intraamniotic infection and/or inflammation in women with placenta previa and vaginal bleeding: an unexpected observation. J Perinat Med. 2010;38:275-279.

Malak TM, Ockleford CD, Bell SC, Dalgleish R, Bright N, Macvicar J. Confocal immunofluorescence localization of collagen types I, III, IV, V and VI and their ultrastructural organization in term human fetal membranes. Placenta. 1993;14:385-406.

Maradny EE, Kanayama N, Halim A, Maehara K, Terao T. Stretching of fetal membranes increases the concentration of interleukin-8 and collagenase activity. Am J Obstet Gynecol. 1996; 174:843-849.

McLean M, Bisits A, Davies J, *et al*. A placental clock controlling the length of human pregnancy. Nat Med. 1995;1:460.

Menon R, Lombardi SJ, Fortunato SJ. TNF-α promotes caspase activation and apoptosis in human fetal membranes. J Assist Reprod Genet.2002;19:201-204.

Menon R, Fortunato SJ. Infection and the role if inflammation in preterm premature rupture of the membranes. Clin Obstet Gynecol.2007;21:467-478.

Menon R, Taylor RN, Fortunato SJ. Chorioamnionitis – a complex pathophysiologic syndrome. Placenta. 2010:31:113-120.

Menon R, Yu J, Basanta-Henry P, Brou L, Berga SL, Fortunato SJ, Taylor RN. Short fetal leukocyte telomere and preterm prelabor rupture of the membranes. PLoSOne.2012;7:1-6.

Menon R. Oxidative stress damage as a detrimental factor in preterm birth pathology. Front. Immunol. 2014;5:1-13.

Moroz LA, Simhan HN. Rate of sonographic cervical shortening and biologic pathways of spontaneous preterm birth. Am J Obstet Gynecol. 2014; 210:555.e1-e5.

Mouithys-Mickalad A, Kohnen S, Deby C, Noels AF, Lamy M, Deby-Dupont G. Peroxynitrite reacts with biological nitrogen-containing cyclic molecules by a radical pathway, as demonstrated by ultraweak luminescence couple with ESR technique. Biochem Biophys Res Commun. 1999; 259:460-464.

Muglia LJ, Katz M. The enigma of spontaneous preterm birth. N Engl J Med. 2010; 362:529-535.

Myatt L. Review: Reactive oxygen and nitrogen species and functional adaptation of the placenta. Placenta. 2010; S66-69.

Murphy K, Travers P, Walport M. Imunologia de Janeway. 7ª Edição. [Porto Alegre]: Artmed; 2010.

Nemeth E, Tashima LS, Yu Z, Bryant-Greenwood GD. Fetal membrane distention: I. Differentially expressed genes regulated by acute distention in amniotic epithelial (WISH) cells. Am J Obstet Gynecol. 2000; 182:50-59.

Newton ER. Preterm labor, preterm premature rupture of membranes and chorioamnionitis. Clin Perinatol. 2005; 32:571-600.

Norwitz ER, Snegovskikh V, Schatz F, Foyouzi N, Rahman M, Buchwalder L, Lee HJ, Funai EF, Buhimschi CS, Buhimschi IA, Lockwood CJ. Progestin inhibits and thrombin stimulates

the plasminogen activator/inhibitor system in term decidual stromal cells: implications for parturition. Am J Obstet Gynecol. 2007; 196:382.e1-e8.

Offenbacher S, Lieff S, Boggess KA, Murtha AP, Madianos PN, Champagne CM, McKaig RG, Jared HL, Mauriello SM, Auten RL Jr, Herbert WN, Beck JD. Maternal periodontitis and prematurity: part I, obstetric outcome of prematurity and growth restriction. Ann Periondontol.2001;6:164-174.

Oh KJ, Lee SE, Jung H, Kim G, Romero R, Yoon BH. Detection of ureaplasmas by the polymerase chain reaction in the amniotic fluid of patients with cervical insufficiency. J Perinat Med. 2010;38:261-268.

Orrenius S, Nicotera P, Zhivotovsky B. Cell Death Mechanisms and Their Implications in Toxicology. Toxicol Sci. 2011:119:3-19.

Osborn BH, Haney AF, Misukonis MA, Weinberg JB. Inducible nitric oxide synthase expression by peritoneal macrophages in endometriosis-associated infertility. Fertil Steril. 2002;77:46-51.

Ou CW, Orsino A, Lye SJ. Expression of connexin-43 and connexin-26 in the rat myometrium during pregnancy and labor is differentially regulated by mechanical and hormonal signals. Endocrinology. 1997;138:5398-5407.

Pacora P, Chaiworapongsa T, Maymon E, Kim YM, Gomez R, Yoon BH, Ghezzi F, Berry SM, Qureshi F, Jacques SM, Kim JC, Kadar N, Romero R. Funisitis and chorionic vasculitis: the histological counterpart of the fetal inflammatory response syndrome. J Matern Fetal Neonatal Med. 2002;11:18-25.

Park CW, Moon KC, Park JS, Jun JK, Romero R, Yoon BH. The involvement of human amnion in histologic chorioamnionitis is an indicator that a fetal and an intra-amniotic inflammatory response is more likely and severe: clinical implications. Placenta. 2009;30:56-61.

Parry S, Strauss JF. Premature Rupture of the Fetal Membranes. N Engl J Med 1998; 338:663-670.

Pinho MSL. Proteina p53: Algum valor clínico ou apenas pesquisa? Uma revisão da literatura. Rev Bras Coloproct. 2000;20:258-260.

Polettini J, Dutta EH, Behnia F, Saade GR, Torloni MR, Menon R. Aging if intrauterine tissues in spontaneous preterm birth and preterm premature of the membranes: A systematic review of the literature. Placenta. 2015:36:969-973.

Ramsey PS, Lieman JM, Brumfield CG, Carlo W.Chorioamnionitis increases neonatal morbidity in pregnancies complicated by preterm premature rupture of membranes. Am J Obstet Gynecol. 2005;192:1162-1166.

Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, Vogler C, Society for Pediatric Pathology, Perinatal Section, Amniotic Fluid Infection Nosology Committee. Amniotic infection syndrome: nosology and reproducibility of placental reaction patterns. Pediatr Dev Pathol. 2003;6:435-448.

Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Biol. 1994;124:1-6.

Reynaert NL, Ckless K, Wouters EF, van der Vliet A, Janssen-Heininger YM. Nitric oxide and redox signaling in allergic airway inflammation. Antioxid Redox Signal. 2005;7:129-143.

Richter C, Park JW, Ames BN. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Natl Acad Sci USA. 1988; 85:6465-6467.

Romero R, Mazor M, Munoz H, Gomez R, Galasso M, Sherer DM. The preterm labor syndrome. Ann M Y Acad Sci. 1994; 734:414-429.

Romero R, Espinoza J, Gonçalves LF, Kusanovic JP, Friel LA, Nien JK. Inflammation in preterm and term labour and delivery. Semin Fetal Neonatal Med. 2006; 14: 317-326.

Romero R, Espinoza J, Kusanovic J, Gotsch F, Hassan S, Erez O, Chaiworapongsa T, Mazor M. The preterm parturition syndrome. BJOG. 2006;113:17- 42.

Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes.Science. 2014;345:760-765.

Romero R, Miranda J, Chaiworapongsa T, Chaemsaithong P, Gotsch F, Dong Z, Ahmed AI, Yoon BH, Hassan SS, Kim CJ, Korzeniewski SJ, Yeo L, Kim YM. Sterile intra-amniotic inflammation in asymptomatic patients with a sonographic short cervix: prevalence and clinical significance. J Matern Fetal Neonatal Med. 2014;24:1-17.

Romero R, Miranda J, Chaiworapongsa T, Chaemsaithong P, Gotsch F, Dong Z, Ahmed AI, Yoon BH, Hassan SS, Kim CJ, Korzeniewski SJ, Yeo L. A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. Am J Reprod Immunol. 2014:71:330-358.

Romero R, Miranda J, Kusanovic JP, Chaiworapongsa T, Chaemsaithong P, Martinez A, Gotsch F, Dong Z, Ahmed AI, Shaman M, Lannaman K, Yoon BH, Hassan SS, Kim CJ, Korzeniewski SJ, Yeo L, Kim YM.Clinical chorioamnionitis at term I: microbiology of the amniotic cavity using cultivation and molecular techniques. J Perint Med. 2015:43:19-36.

Romero R, Gomez R, Chaiworapongsa T, Conoscenti G, Kim JC, Kim YM.The role of infection in preterm labour and delivery. Paediatr Perinat Epidemiol. 2001;15:41-56.

Rosen T, Schatz F, Kuczynski E, Lam H, Koo AB, Lockwood CJ. Thrombin-enhanced matrix metalloproteinase-1 expression: a mechanism linking placental abruption with premature rupture of the membranes. J Matern Fetal Neonatal Med 2002; 11:11-17.

Rosselli M, Keller PJ, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update. 1998;4:3-24.

Salafia CM, López-Zeno JA, Sherer DM, Whittington SS, Minior VK, Vintzileos AM. Histologic evidence of old intrauterine bleeding is more frequent in prematurity. Am J Obstet Gynecol. 1995; 173:1065-1070.

Schrier RW, Wang W. Acute renal failure and sepsis. N Engl J Med. 2004;351:159-169.

Sies H. Oxidative stress: from basic research to clinical application. Am J Med. 1991; 91: 31S-38S.

Sies H. Strategies of antioxidant defense. Eur J Bio. 1993; 215:213-219.

Sperling RS, Newton E, Gibbs RS. Intra-amniotic infection in low-birth-weight infants. J Infect Dis. 1988;157:113-117.

Sooranna SR, Engineer N, Loudon JA, Terzidou V, Bennett PR, Johnson MR. The mitogen-activated protein kinase dependent expression of prostaglandin H synthase-2 and interleukin-8 messenger ribonucleic acid by myometrial cells: the differential effect of stretch and interleukin-1{beta}. J Clin Endocrinol Metab. 2005; 90:3517-3527.

Subash P, Gurumurthy P,Sarasabharathi A, Cherian KM. Urinary 8-OHdG: a marker of oxidative stress to DNA and total antioxidant status in essential hypertension with South Indian population. Indian J Clin Biochem. 2010; 25:127-132.

Swamy GK, Ostbye T, Skjaerven R. Association of preterm birth with long-term survival, reproduction, and next-generation preterm birth. JAMA. 2008;299:1429–1436.

Vaisbuch E, Hassan SS, Mazaki-Tovi S,Nhan-Chang CL, Kusanovic JP, Chaiworapongsa T, Dong Z, Yeo L, Mittal P, Yoon BH, Romero R. Patients with an asymptomatic short cervix (< or = 15mm) have a high rate of subclinical intraamniotic inflammation: implications for patient counseling. Am J Obstet Gynecol. 2010;202:433.e1-e8.

Valgimigli L, Pedulli GF, Paolini M. Measurement of oxidative stress by EPR radical-probe technique. Free Radic Biol Med. 2001; 31:708-716.

Vega M, Johnson MC, Diaz HA, Urrutia LR, Troncoso JL, Devoto L. Regulation of human luteal steroidogenesis in vitro by nitric oxide. Endocrine. 1998;8:185-191.

Vousden KH, Lu X. Live or let die: The cell's response to p53. Nature. 2002;2:594-604.

Wladimiroff JW, Campbell S. Fetal urine-production rates in normal and complicated pregnancy. Lancet. 1974;2:151-154.

Webster RP, Roberts VHJ, Myatt L. Protein Nitration in Placenta - Functional Significance. Placenta. 2008; 29:985-994.

Word RA, Stull JT, Casey ML, Kamm KE. Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. J Clin Invest. 1993; 92:29-37.

Capítulo II

Artigo Científico I

8-OXO-2'-DEOXIGUANOSINE LEVELS IN AMNIOCHORION MEMBRANES FROM PREGNANCIES COMPLICATED BY PREMATURITY

Moisés D. de Lima[1], Laura F. Martin[2], Natália P. Moço[2], Andressa R. Olah[2], Márcia G. da Silva[2*]

[1.] Department of Obstetrics and Gynecology, Health Sciences Center, Federal University of Paraíba, UFPB, João Pessoa, Brazil.

[2.] Department of Pathology, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil.

*Corresponding author

Department of Pathology, Botucatu Medical School, São Paulo State University – UNESP

Distrito de Rubião Júnior, Zip Code 18618-970, Botucatu, São Paulo, Brazil.

Phone: +55 (14) 3880 1580 Fax: +55 (14) 3815-2348

E-mail: mgsilva@fmb.unesp.br

ABSTRACT

Objective: To evaluate the levels of 8-oxo-2'-deoxiguanosine (8-OHdG) in amniochorion membranes from pregnancies complicated by prematurity. **Methods:** In this cross-sectional study, were enrolled 31 with Preterm Labor and intact membranes (PTL) and 35 with Premature Rupture of Preterm Ovular Membranes (pPROM) who presented preterm delivery. As control group was included 37 pregnant women that delivery at term. Amniochorion membranes were collected and total DNA extraction was performed by ILLUSTRA tissue &cells genomic-Prep Mini Spin Kit and 8-OHdG levels were measured by an ELISA Highy Sensitive 8-OHdG Check kit. The distribution of the data was checked by Kolmogorov-Smirnov normality test and comparisons between the groups were performed using non-parametric Mann-Whitney and Kruskal-Wallis tests. Statistical significance was considered at $p<0.05$ **Results:** Regarding to data, only gestational age at delivery and newborn weight at birth were statistically higher in term group when compared to PTL and pPROM groups. 8-OHdG levels in amniochorion membranes of term group (2,90 ng/mL [1,54 – 4.06]) were significantly higher than preterm labor group (0,61 ng/mL [0,37 – 0,92]) ($p<0.001$). 8-OHdG levels were also higher in term group than in PTL (0,71 ng/mL [0,40 – 1,47]) or pPROM groups (0,53 ng/mL [0,37 – 0,71]) ($p<0.001$). **Conclusion:** Our data reinforces that oxidative damage are present at term pregnancies as physiologic process of amnionchorion aging.

Keywords: 8-OHdG, Preterm Labor, Preterm Premature Rupture of Membranes.

INTRODUCTION

Reactive Oxygen Species (ROS) are generated as metabolites of cellular respiration[1] and participate in several functions such as cellular signaling[2], defense against pathogens[3], activation of transcription factors and cell reproduction[4]. Elevated ROS levels may cause extensive oxidative lipids, protein and DNA damage[2]. Oxidative stress is referred to as an imbalance between the generation of ROS and their clearance by defensive antioxidants[5].

During pregnancy, the multiple changes that occur in maternal organism culminate in an increase in the baseline level of oxygen, which characterizes the gestational period as susceptible to oxidative stress[6]. Although the fetoplacental unit generates abundant antioxidants in order to keep the oxidative stress under control, the increase of ROS during the gestational period has been associated with several complications in pregnancy as preeclampsia[7]first-trimester spontaneous abortion[8.9]and premature rupture of preterm ovular membranes (pPROM)[10].

Oxidation caused by high levels of ROS can lead to cell genotoxity and genomic instability[11]. Guanine-rich sequences, such as the telomere sequence, present a higher reactivity compared to those who have a single guanine[12]. Telomere sequence consists in a repetition of six nucleotides (TTAGGG) what makes this region more susceptible to oxidative stress than the rest of the genome[13]. Among the multiple lesions caused by ROS that may occur in the telomere region, 8-hydroxylation of guanine is one of the most common[14] causing a transverse mutation exchanging GC for TA. Accumulation of DNA single strand breaks in the region of the telomeres occurs when the lesion 8-oxoG is not safe repaired by the 8-oxoguanine glycosylase (OGG1) leading to its shortening and senescence[15]. In pregnancy, lower levels of gene and protein expression of OGG1 were found in fetal membranes from pPROM cases in comparison to spontaneous preterm birth (PTB)[16].

It has been established that cases of pPROM has a similar outcome to the parturient at term, especially in regard to factors such as degradation of membranes, apoptosis and signs of oxidative damage[17,18,19]. Oxidative damage may cause either direct effects in the

mechanisms of birth and the rupture of the membranes or indirect effectsin the inflammatory mediators.

Elevated ROS levels in amnion cells may induce PTB and pPROM via senescence associated secretory phenotype (SASP)[20], a phenotype that has the capacity of turn senescent cells in proinflammatory cells[11].

Telomere shortening cause by 8-hydroxylation of guanine can lead to cellular senescence of membranes and eventual rupture. Larger telomeres in cases of PTB when compared with cases of pPROM and parturient at term sustained this affirmation[17].

Therefore, considering a possible correlation between oxidative stress and adverse gestational outcomes, the aim of this study was evaluate the levels of 8-oxo-2'-deoxiguanosine (8-OHdG) in amniochorion membranes from pregnancies complicated by prematurity, including PTB in labor (PTL) and pPROM.

MATERIAL AND METHODS

Study population

A cross-sectional study was conducted in the Obstetrics Unit of the Lauro Wanderley Hospital, Federal University of Paraíba (UFPB), João Pessoa, Paraíba State, Brazil and in Botucatu Medical School, São Paulo State University, UNESP. A total of 103 pregnant women participated in this study. The 66 women that delivered preterm, including 31 PTL with intact membranes and 35 pPROM were recruited for this study between January 2014 and December 2015. As a control group, 37 health pregnant women that delivered at term were included. Women were eligible if they were having a singleton term and uneventful pregnancy defined as being free from any chronic or gestational medical conditions, including any condition that would require activity restrictions, intact membranes and irrelevant obstetric history.

Gestational age was calculated from the first day of last menstrual period and/or by first-trimester ultrasound examination. Clinical diagnoses of PTL were made with the

following criteria: Uterine contractions of the frequency ≥4 per 20 minutes, cervical dilatation of at least 1 cm and cervical ripening (21). Rupture of fetal membranes was confirmed with vaginal discharge pH ≥7 and/or positive result of Amni Sure test[22].

Exclusion criteria for the groups were pregnant women with BMI<25 kg/m^2, preeclampsia, HELLP syndrome, gestational diabetes, hypertension, multiple pregnancies, cervical isthmus incompetence, placenta previa, placental abruption, RH-incompatibility, oligamnios or polyhydramnios, intrauterine growth restriction, malformation or fetal deaths, systemic infection, thyroid disease, HIV infection and drug users.

The research project was approved by the Ethics Committee in Research of UFPB (Protocol: 1.806.905) and written informed consent was obtained from all the participants.

Measurement of 8-OHdG levels

Amniochorion membranes were collected in sterile conditions after placenta expelling and immediately frozen in liquid nitrogen and stored at -80°C until processing.

Total DNA extraction was performed with ILLUSTRA tissue & cells genomic-Prep Mini Spin Kit (GE Healthcare, Little Chalfont, UK), in accordance with manufactured instructions. DNA samples were denatured at 95°C for 3 minutes in TermoMixer Comfort (Eppendorf, Hamburg, Germany). The total extracted DNA was quantified in the spectrophotometer absorbance of 260nm and purity was determined by the ratio of the absorbance 260nm/absorbance 280nm (A_{260}/A_{280}). Reasons higher than 1.75 were considered acceptable. Samples had DNA concentrations adjusted to 50ng/µL.

For the enzymatic digestion of DNA were added 2.5 µL of sodium acetate (20 mM), followed by the addition of 1.5 units of nuclease P1 (USBiological, Salem, USA) in each sample. The samples were incubated at 37°C for 30 minutes in TermoMixer Comfort (Eppendorf, Hamburg, Germany). After that, it was added 0.5 unit of alkaline phosphatase (Thermo Scientific, Massachusetts, USA) and the samples were incubated at 37° C for 10 minutes in TermoMixer Comfort (Eppendorf, Hamburg, Germany). The hydrolysates were

filtered through Millipore Microcon YM 10 columns (Millipore, Massachusetts, USA) for 10 minutes at 14000 RPM to remove enzymes and other macromolecules.

8-OHdG levels were measured by an ELISA Highy Sensitive 8-OHdG Check kit (JaICA, Shizuoka, Japan), according to the manufacturer's instructions. A volume of 50µL 8-OHdG standard was used for the assay. A standard curve was obtained in parallel to each assay and the absorbance results were converted to ng/mL. At the end of reaction, the absorbance was read spectrophotometrically at 450nm in an automatic ELISA reader (Biotek Instruments Inc, Winooski, USA) and the concentration of 8-OHdG in each sample were determined by comparison against a standard curve. All the samples were tested in duplicate. The minimum detectable 8-OHdG level for assays was 0.031ng/mL.

Statistical Analysis

The Kolmogorov-Smirnov test was used to check the normality of the data. Regarding sociodemographic and obstetrics variables, ethnicity was compared among the groups using Fisher's Exact Test, while the variable maternal age was compared by the ANOVA. The variables gestational age at delivery and newborn weight were compared among PTL, pPROM and term groups using Kruskal-Wallis test.

Regarding to 8-OHdG levels, comparison between preterm group and term group were made using Mann-Whitney test. Comparison of 8-OHdG levels among PTL, pPROM and term groups were made using Kruskal-Wallis test. Statistical analysis were conducted using statistical software SigmaStat 3.5 and statistical significance was considered at $p<0.05$.

RESULTS

Sociodemographic characteristics of the pregnant women included in the study are presented in Table 1. There were no statistical differences in maternal age, ethnicity and parity among the groups. As expected due to the study design, gestational age at delivery ($p<0.001$) and newborn weight ($p<0.001$) were statistically higher in term group when compared to PTL and pPROM groups.

Table 1. Characteristics of the pregnant women included in the study.

Characteristics		Term (n=37)	PTL (n=31)	pPROM (n=35)	p-value
Maternal age (years)*		23.650 ± 6,604a	24.35±6,002a	26.1 ± 6,703a	NS
Ethnicity	**White**	15% (3/20)a	30% (6/20)a	30% (6/20)a	NS
	Non-White	85% (17/20)a	70% (14/20)a	70% (14/20)a	NS
Gestational age at delivery (days)		273 (259-294)a	240.5 (165-256)b	245 (179-252)b	<0.001
Parity	**=1**	40%(8/20)a	40% (8/20)a	35% (7/20)a	NS
	>1	60(12/20)a	60% (12/20)a	65% (13/20)a	NS
Newborn weight (grams) * *		3257.5 (2825-3600)a	2272.5 (630-3085)b	2305 (740-2980)b	<0.001

* values expressed as mean ± SD
* *values expressed as median (min/max)
NS: Not significant

Regarding 8-OHdG, the term group (2,90 ng/mL [1,54 – 4.06]) amniochorion membranes exhibited significantly higher levels than preterm group (0,61 ng/mL [0,37 – 0,92]) (p<0.0001). Similarly, 8-OHdG levels in term group were higher than in PTL (0,71 ng/mL [0,40 – 1,47]) (p<0.0001) and pPROM groups (0,53 ng/mL [0,37 – 0,71]) (p<0.0001) (Figure 1).

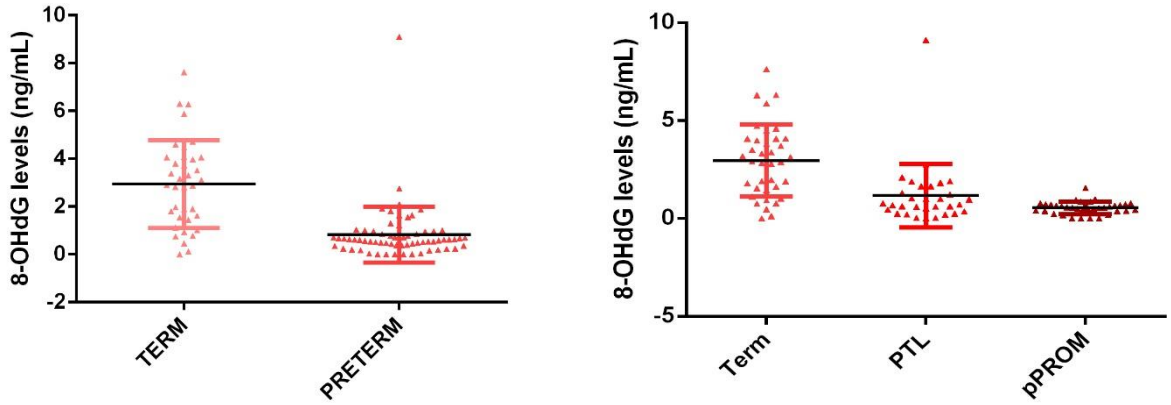


Fig 1. (A) 8-OHdG levels in amniochorion membranes from term and preterm groups. * Mann-Whitney test. *p*< 0,001. **(B)**8-OHdG levels in amniochorion membranes from term, preterm labor with intact membranes (PTL) and premature rupture of preterm ovular membranes (pPROM). **Kruskal-Wallis test. *p*< 0.001.

DISCUSSION

Given the considerable burden imposed by prematurity on the maternal-fetal health, a great deal of research has been conducted to understand the mechanisms and the risk factors that are involved in preterm delivery.

There are many explanations for the ROS contribution in cases of preterm birth such as upregulation of proinflammatory mediators that initiate labor[23] collagen degradation of chorioamniotic membranes resulting in membrane rupture[10]and also nucleotide oxidation leading to senescence and pPROM[18].

Pregnancy outcome of parturient at term and pPROM have similarities such as membrane degradation and rupture before labor, apoptosis and elevated ROS levels[17,18,19]. Previous research demonstrated shorter telomeres in parturient at term and pPROM when compared with PTB cases[17,19]. However, there is a lack of solid evidence for the associationbetween placental aging and PTB and/or pPROM due to thescarcity of studies on this topic[20].

Contrary to expected, our results demonstrated that 8-OHdG levels in amniochorion membranes from term pregnancies were significantly higher in comparison with pPROM membranes. Similarly, 8-OHdG levels in term group were higher than in PTL membranes.

A possibleexplanation for lower 8-OHdG levels found in pPROM group when compared with term group may be correlated with the fact that 8-OHdG levels progressively increase as pregnancy progress. The aging of term placental tissues is a physiologic phenomenon. As pregnancy advances, the productionROS increases due to the highermetabolic demands of the growing fetus and maximum production occurs prior to term delivery[21,22]. Ferguson et al.[23] measure 8-OHdG levels in urine of pregnancy woman at 10,18, 26 and 35 weeks and verify that they increased in a quadratic form according with the progress of pregnancy.

Another point to consider is the concept of sterile inflammation has recently arise in the pregnancy context. One third of pPROM pregnancies present sterileintra-amniotic

inflammation[24]evidencing that microbialintraamniotic infection is not the only source of inflammation. In this line, Behnia et al.[25]have suggested two independently pathways for prematurity considering the presence of infection or oxidative stress.In this work, the authors demonstrated NF-κB activation, apoptosis and minimal DNA damage of amnion cells after LPS-stimulation, mimicking intraamniotic infection. On the other hand, cigarette smoke extract mimicking oxidative stress has driven generation of 8-OHdG, telomere attrition and sterile inflammation. In accordance, maternal smoking increases the level of the oxidative DNA damage biomarker 8-OHdG in umbilical cord[26].Therefore, a limitation of this study is that we did not consider the histologic chorioamnionitis status in preterm membranes, a well-document feature of intraamniotic infection.Seventy per cent of our preterm samples presented chorioamnionitis, which may have influenced our results. The subgroup analysis was not feasible as the sample size is limited.

Further studies evaluating combined comparison of 8-OHdG levels, biomarkers of senescence and proinflammatory mediators throughout the last weeks of pregnancy and considering the histologic chorioamnionitis status are necessary to better understanding of the possible correlation between 8-OHdG levels and preterm delivery.

CONCLUSION

Present data show that 8-OHdG are higher in parturient at term than women with pregnancies complicated by PTL or pPROM. It reinforces that oxidative damage are present at term pregnancies as physiologic process of amnionchorion aging.

REFERENCES

1. Sokhansanj BA, Wilson 3rd DM. Estimating the effect of human base excision repair protein variants on the repair of oxidative DNA base damage. Cancer Epidemiol Biomarkers Prev 2006;15:1000–1008.
2. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 2000; 279: L1005 - L1028.
3. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44–84.

4. Agarwal A, Gupta S, Sekhon L, Shah R. Redox Considerations in Female Reproductive Function and Assisted Reproduction : From Molecular Mechanisms to Health Implications. Antioxid Redox Signal 2008;10:1375-1403.
5. Duracková Z. Some Current Insights into Oxidative Stress 2010;59:459–469.
6. Casanueva E, Viteri FE. Iron and Oxidative Stress in Pregnancy. J Nutr 2003;133:1700S–1708S.
7. Biron-Shental T, Sukenik-Halevy R, Sharon Y, Goldberg-Bittman L, Kidron D, Fejgin MD, et al. Short telomeres may play a role in placental dysfunction in preeclampsia and intrauterine growth restriction. Am J Obstet Gynecol 2010; 202:e1-e7.
8. Jauniaux E, Watson AL, Hempstock J, Bao Y, Skepper JN, Burton GJ. Onset of Maternal Arterial Blood Flow and Placental Oxidative Stress. A Possible Factor in Human Early Pregnancy Failure. Am J Pathol 2000;157:2111–2122.
9. Jauniaux E, Greenwold N, Hempstock J, Burton GJ. Comparison of Ultrasonographic and Doppler Mapping of the Intervillous Circulation in Normal and Abnormal Early Pregnancies. Fertil Steril 2003;79: 100-106.
10. Woods Jr JR. Reactive Oxygen Species and Preterm Premature Rupture of Membranes - A Review. Placenta 2001; 22: S38–S44.
11. Xu B, Wang W, Guo H, Wei Z, Zhang X, Liu Z , et al. Oxidative Stress preferentially induces a subtype of micronuclei and mediates the genomic instability caused by p53 dysfunction. Mutat Res 2014; 770: 1-8.
12. Coluzzi E, Colamartino M, Cozzi R, Leone S, Meneghini C, O´Callaghan N, et al. Oxidative Stress Induces Persistent Telomeric DNA Damage Responsible for Nuclear Morphology Change in Mammalian Cells. PloS One 2014;9: e110963.
13. Henle ES, Han Z, Tang N, Rai P, Luo Y, et al. Sequence-Specific DNA Cleavage by Fe2+ Mediated Fenton Reactions has Possible Biological Implications. J Biol Chem 1999;274:962–71.
14. Xu Z, Duc KD, Holcman D, Teixeira MT. The Length of the Shortest Telomere as the Major Determinant of the Onset of Replicative Senescence. Genetics 2013;194:847–857.
15. Blackburn EH. Switching and Signaling at the Telomere. Cell 2001;106:661–673.
16. Menon R, Polettini J, Syed TA, Saade GR, Boldogh I. Expression of 8-Oxoguanine Glycosylase in Human Fetal Membranes. Am J Reprod Immunol 2014; 72:75-84.
17. Menon R, Yu J, Basanta-Henry P, Brou L, Berga SL, Fortunato SJ, et al. Short Fetal Leukocyte Telomere Length and Preterm Prelabor Rupture of the Membranes. PLoS One 2012; 7: e31136.
18. Menon R. Oxidative Stress Damage as a Detrimental Factor in Preterm Birth Pathology. Front Immunol 2014; 5:567.
19. Menon R, Boldogh I, Hawkins HK, Woodson M, Polettini J, Syed TA, et al. Histological Evidence of Oxidative Stress and Premature Senescence in Preterm Premature Rupture of the Human Fetal Membranes Recapitulated In Vitro. Am J Pathol 2014; 184: 1740-1751.
20. Polettini J, Dutta EH, Behnia F, Saade GR, Torloni MR, Menon R. Aging of Intrauterine Tissues in Spontaneous Preterm Birth and Preterm Premature Rupture

of the Membranes: A Systematic Review of the Literature. Placenta 2015; 36: 969 – 973.

21. Al-Gubory KH, Fowler PA, Garrel C. The Roles of Cellular Reactive Oxygen Species, Oxidative Stress and Antioxidants in Pregnancy Outcomes. Int J Biochem Cell Biol 2010;42:1634-1650.

22. Myatt L, Cui X. Oxidative Stress in the Placenta. Histochem Cell Biol 2004;122:369-382.

23. Ferguson KK, McElrath TF, Chen Y, Loch-Caruso R, Mukherjee B, Meeker JD. Repeated Measures of Urinary Oxidative Stress Biomarkers during Pregnancy and Preterm Birth. Am J Obstet Gynecol 2015;212:208.e1–e8.

24. Romero R, Miranda J, Chaemsaithong P, Chaiworapongsa T, Kusanovic JP, Dong Z.Sterile and Microbial-Associated Intra-Amniotic Inflammation in Preterm Prelabor Rupture of Membranes. J Matern Fetal Neonatal Med 2015; 28:1394–1409.

25. Behnia F, Sheller S, Menon R. Mechanistic Differences Leading to Infectious and Sterile Inflammation. Am J Reprod Immunol 2016; 75: 505–518.

26. Satoko E, Takako C, Takashi O, Ikuo K. Relationship Between 8-Hydroxydeoxyguanosine Levels in Placental/Umbilical Cord Blood and Maternal/Neonatal Obstetric Factors. Exp Ther Med 2012; 4:387–390.

Artigo Científico II

p53 PROTEIN EXPRESSION IN AMNIOCHORION MEMBRANES: ASSOCIATION BETWEEN HISTOLOGIC CHORIOAMNIONITIS, OXIDATIVE STRESS, AND PRETERM BIRTH

Moisés D. de Lima[1*], Alexandre R. da Paz[2], Laura F. Martin[3], Márcia G. da Silva[3]

[1.] Department of Obstetrics and Gynecology, Health Sciences Center, Federal University of Paraíba, UFPB, João Pessoa, Brazil.

[2.]Department of Surgery, Medical Sciences CenterHealth Sciences Center, Federal University of Paraíba, UFPB, João Pessoa, Brazil.

[3.] Department of Pathology, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil.

*Corresponding author

Department of Obstetrics and Gynecology, Health Sciences Center,

Federal University of Paraíba, UFPB, João Pessoa, Brazil

Phone number: +55 (83) 3216-7041

E-mail: drmoiseslima@gmail.com

ABSTRACT

Introduction: Etiology of preterm delivery is not fully understood and preterm birth remains responsible for the high rates of perinatal morbidity and mortality. Infection of the amniotic cavity and chorioamnionitis, are certainly the most studied mechanisms, being manifested by an excessive production of inflammatory mediators. Other mechanisms, such as oxidative stress and apoptosis, may also contribute for this pregnancy adverse outcome. Guanine, whose DNA oxidation product is represented by the 8-oxo-2'-deoxyguanosine (8-OHdG) is the nucleotide base most susceptible to changes induced by free radicals. Apoptosis is another route possibly related to preterm birth, being characterized by a rapid progression of degenerative cellular phenomena. Its mechanisms may also be related to DNA damage and infection. **Objective:** The aim of this study was to analyze the prevalence of the histological chorioamnionitis, oxidative stress (8-OHdG), and p53 protein expression (apoptosis) in amniochorion membranes in women affected by premature rupture of preterm ovular membranes (pPROM) and preterm labor (PTL) and investigate which of these events is most associated with apoptosis occurrence. **Methods**: It was a prospective study conducted in the Obstetrics Unit of the Lauro Wanderley University Hospital (HULW), Federal University of Paraíba (UFPB), Paraiba State, Brazil from January to December 2014 and a total of 60 pregnant women were enrolled, being 31 pregnant women who presented pPROM and 29 with PTL. After delivery, the amniochorion membranes were subjected to a histopathological examination, to 8-OHdG levels analysis by an ELISA Highy Sensitive 8-OHdG Check kit and occurrence of apoptosis by p53 protein immunohistochemical study. **Results**: Histologic chorioamnionitis was detected in 64.5% of the amniochorion membranes from pPROM group and in 44.8% from PTL group (p=0.12). The 8-OHdG levels in the PTL group [0.71 (min: 0.43 – max: 1.38)] were significant higher than in the pPROM group [0.53 (min: 0.37- max: 0.69)] (p=0.02). The occurence of apoptosis in the pPROM group (32.2%) was similar in the PTL group (24.1%) (p=0.48). Logistic regression analysis among these mechanisms showed that only histologic chorioamnionitis is related to apoptosis (OR=3.7; 95%CI: 1.19-14.9; p=0.04). **Conclusion**: Among the factors studied, histologic chorioamnionitis seems to be more clearly associated with apoptosis occurrence in amniochorion membranes from pregnancies complicated by spontaneous prematurity.

Keywords: Preterm birth, histologic chorioamnionitis, apoptosis, 8-oxo-2'-deoxyguanosine.

INTRODUCTION

Preterm birth has variable incidence, in the range 5-18% of all pregnancies [1]. Its etiology is not fully understood, and thus its occurrence rates continuerising in the last three decades. Furthermore, preterm birth also remains as responsible for the high rates of perinatal morbidity and mortality [2,3].

Infection of the amniotic cavity designates the state in which this inflammatory process occurs. It´s characterized by involvement of the amniochorion membranes, amniotic fluid, placenta, and/or maternal decidua [4], being a risk factor strongly related to the maternal and neonatal adverse outcomes [5]. Chorioamnionitis is caused by several bacterial species, being certainly one of the most studied mechanisms. It occurs by excessive production of inflammatory mediators which act in the gestational tissues and contribute with the events that lead to the preterm birth [6].

It is likely that other mechanisms participate in the inflammatory environment and form alternative and distinct molecular pathways still little known. Among these mechanisms, the oxidative stress and apoptosis are currently hypotheses associated with the physiopathology of the preterm birth.

A complex respiratory mechanism is necessary to supply power to the cell. It allows a residual release of reactive oxygen species (ROS), which react with other organic constituents via subsequent molecular reactions [7]. In contrast to the production of ROS, which are physiologically produced, the cells need to maintain a stable balance between them and the antioxidant antagonist mechanisms [8,9]. Repair of the cumulative oxidant effects observed in the cells may occur by prevention and elimination of free radicals produced as well as fixing already damaged molecules [10]. Therefore, oxidative stress (OS) results from this imbalance, being one of the mechanisms capable of causing damage to cellular macromolecules such as lipids, proteins and DNA [7,11].

In-vivo measurement of free radicals is possible and their detection ismore specific when multiple markers are investigated. They should ideally be analyzedin tissues or fluids

supposed to have suffered oxidative damage [9,12]. Among them, DNA damage is molecularly characterized by breaks in their single or double chains, exchanges between sister chromatids, DNA crosslinking or even changes in nitrogenous bases [13,14]. Guanine is the nucleotide base most susceptible to free radicals-induced changes. Its DNA oxidation product is 8-hydroxydeoxyguanosine (8-OHdG), which clearly indicates a pathological process when in high concentrations, with genomic instability that leads to cell death [15].

Apoptosis is another possible route related to preterm birth, being characterized by a rapid progression in the cellular degenerative phenomena. Its mechanisms may also be related to DNA damage triggered by the oxidative stress, infection, or other factors not yet fully understood [2, 6, 16 - 18].

To the apoptotic process, a complex cascade of biochemical reactions installs within the cell. One of the ways is related to the expression of p53 protein, a molecule encoded by the *p53* gene, so named because of its molecular weight (53 kDa) [19]. Its main function is related to integrity preservation of the genetic code in each cell, i.e., maintaining the same nucleotide sequence along all the DNA molecule. This gene is located on chromosome 17 and is also present in all cells of the human body [20].

During the cell division cycle, the p53 protein verifies any occurrence of mutation in the genetic code sequence. If the existence of a mutation is detected, p53 protein participatesin a reaction cascade and prevents the cell from entering the mitosis process and completing cell division. For this purpose, two biochemical pathways can be activated, mutation correction by repair protein activation or induction of cell death by apoptosis, especially in situations of chronic stress with recurrent DNA damage [21].

Pregnancy is a period favorable for such imbalance, due to metabolic peculiarities that occurin gestational tissues. This occurs alone or together, triggering the pathways that lead topremature birth, notably the phenotypes associated with preterm premature rupture of membranes (pPROM) and preterm labor (PTL) [22-24].

The aim of this studywas to analyze the association among histologic chorioamnionitis, apoptosis occurence and 8-OHdG levels in amniochorion membranes from

pregnancies complicated by preterm premature rupture of membranes (pPROM) and preterm labor (PTL).

MATERIAL AND METHODS

It was a prospective study conducted in the Obstetrics Unit of the Lauro Wanderley University Hospital (HULW), Federal University of Paraíba (UFPB), Paraiba State, Brazil from January to December 2014 and a total of 60 pregnant women were enrolled, being 31 pregnant women who presented pPROM and 29 with PTL. The groups were between 23 and 36 gestational weeks confirmed by both date of last menstruation and obstetric echography performed up to the 12 week of pregnancy.

Diagnosis of pPROM was considered when spontaneous loss of amniotic fluid occurred, as visualized by physical examination. This examwas performedusing a sterile vaginal speculum before the patients have completed 37 weeks of gestation, and confirmed by the Amni Sure test. The presence of regular uterine contractions (3/10 min) and cervical dilation (≥3 cm) were the definition of PTL.

Women with preeclampsia, HELLP syndrome, placenta previa, placental abruption, restriction in intrauterine growth, cervical incompetence, oligohydramnios, polyhydramnios, fetal malformation, stillbirth, HIV-AIDS, gestational diabetes, Rh incompatibility, and illicit drug users were excluded.

Information on maternal age, body mass index (BMI; as defined by the World Health Organization, WHO), ethnicity (self-reported), parity, gestational age, and newborn weight were obtained in the medical records to compose the general characteristics in the groups analyzed.

During the hospitalization period, all women participating in the study were assisted according to the clinical protocols of medical service at the HULW-UFPB. The research projectwas approved by the Ethics Committee Board in Research of UFPB (Protocol # 1.806.905) and written informed consent was obtained from all the participants.

Histopathological analysis of the amniochorion membranes

After birth, the placenta and ovular membranes were collected under sterile conditions, and fragments near the region of fetal membrane rupture were sectioned and fixed in 10% formalin. Subsequently, the samples were submitted to histopathological analysis by the hematoxylin-eosin technique, and the diagnosis was madeby a single examiner, without prior knowledge of clinical conditions related to the case.

The histopathologic criteria adopted for the diagnosis of chorioamnionitis was established by Redline et al., 2003 and Society for Pediatric Pathology, Perinatal Section, Amniotic Fluid Infection Nosology Committee, considering the presence of maternal inflammatory response in the chorioamniotic membranes in the situations as follows: acute subchorionitis or early acute chorionitis, characterized by the presence of neutrophils in the subchorionic and/or chorionic spaces, (**stage 1)**; acute chorioamnionitis, characterized by neutrophil infiltration in the chorionic connective tissue and/or chorionic plate, (**stage 2)**; and necrotizing chorioamnionitis, a necrosis that reaches the amnion, (**stage 3)**. Regarding degree: the group of maternal neutrophils diffusely found in the chorionic plate, chorium, or amnion were established as mild-to-moderate ([**grade 1**]) and presence of at least three microabscesses was established as severe (**grade 2**) [25].

Measurement of 8-OHdG levels

Fragments of the amniochorion membranes were collected in sterile conditions after placenta expelling and immediately frozen in liquid nitrogen and stored at -80°C until processing.

Total DNA extraction was performed with ILLUSTRA tissue & cells genomic-Prep Mini Spin Kit (GE Healthcare, Little Chalfont, UK), in accordance with manufactured instructions. DNA samples were denatured at 95°C for 3 minutes in TermoMixer Comfort (Eppendorf, Hamburg, Germany). The total extracted DNA was quantified in the spectrophotometer absorbance of 260nm and purity was determined by the ratio of the absorbance

260nm/absorbance 280nm (A_{260}/A_{280}). Reasons higher than 1.75 were considered acceptable. Samples had DNA concentrations adjusted to 50ng/µL.

For the enzymatic digestion of DNA were added 2.5 µL of sodium acetate (20 mM), followed by the addition of 1.5 units of nuclease P1 (USBiological, Salem, USA) in each sample. The samples were incubated at 37°C for 30 minutes in TermoMixer Comfort (Eppendorf, Hamburg, Germany). After that, it was added 0.5 unit of alkaline phosphatase (Thermo Scientific, Massachusetts, USA) and the samples were incubated at 37° C for 10 minutes in TermoMixer Comfort (Eppendorf, Hamburg, Germany). The hydrolysates were filtered through Millipore Microcon YM 10 columns (Millipore, Massachusetts, USA) for 10 minutes at 14000 RPM to remove enzymes and other macromolecules.

8-OHdG levels were measured by an ELISA Highy Sensitive 8-OHdG Check kit (JaICA, Shizuoka, Japan), according to the manufacturer's instructions. A volume of 50µL 8-OHdG standard was used for the assay. A standard curve was obtained in parallel to each assay and the absorbance results were converted to ng/mL. At the end of reaction, the absorbance was read spectrophotometrically at 450nm in an automatic ELISA reader (Biotek Instruments Inc, Winooski, USA) and the concentration of 8-OHdG in each sample were determined by comparison against a standard curve. All the samples were tested in duplicate. The minimum detectable 8-OHdG level for assays was 0.031ng/mL.

Immunohistochemistry for p53 protein

The immunohistochemical study of the anti-p53 protein (Bp53-11; Roche Diagnostics; Indianapolis, IN, USA) was performed by using a technique with the automatedBenchMark ULTRAequipment and Ventana (Ventana Medical Systems, Inc.; Tucson, Arizona, USA) detection kits. Sample slides weredeparaffinized through a series of xylene, alcohol-water gradient, and appropriate buffer washings. The antigen unmasking procedure was performed, and the slides were transferred to the 1X APK wash. The primary antibody (Bp53-11) and the appropriate detection kit dispensers and required accessory reagents onto

the reagent tray and place them on the automated slide stainer. Each step in the staining protocol included incubation for a precise time and at a specific temperature. At the end of each incubation step, the sections were rinsed in the Ventana BenchMark ULTRAautomated slide stainer to stop the reaction and remove the unbound material that would hinder the reaction course in the subsequent steps. To minimize evaporation of aqueous reagents from the specimen-containing slide, a coverslip solution was applied to the slide stainer. Staining was completed after incubation with a substrate chromogen, and the counterstaining (hematoxylin; 2-4 min) and post-counterstain (2-4 min) steps. The stained slides were read within two-to-three days of staining.

Detection of cells in the amniochorion membranes, stained by immunohistochemistry technique was performed by a single pathologist, without prior knowledge of the clinical data related to that sample. The p53-positive immunostaining was defined as being apoptosis diagnosis, when at least ten cells stained by the method were found in seven high-magnification fields, regardless of label immunolocalization and the presence of morphologic changes of the cells related with apoptosis status.

Statistical analysis

Statistical analyzes were performed by using the SigmaStat 3.5 Software (Copyright©2006 Systat Software, Inc. San Jose, CA, USA). Continuous variables were presented as medians and minimum and maximum values and analyzed by using the Mann-Whitney U-test. Categorical variables were analyzed by Chi-squared test, being presented as proportions when necessary. A logistic regression model, which was performed by using the public-domain R (Free Software Foundation's GNU General Public License) software was used to calculatethe adjusted odds ratio for apoptosis occurrence for histologic chorioamnionitis, 8-OHdG levels and obstetric pathologies (pPROM and PTL). The p value < 0.05 was considered statistically significant.

RESULTS

General characteristics of the patients included in the study in pPROM and PTL groups are presented in Table 1. Similar results were observed for maternal age, body mass index, maternal ethnicity and gestational age at delivery.

Table 1. General characteristics of the pregnant women included in the study.

	pPROM (n=31)	PTL (n=29)	*P*
Maternal age* (years)	27.0 (19.2 – 32.0)	22.5 (19.0 – 24.0)	0.07
BMI*	26.4 (23.8 – 29.3)	25.2 (23.4 -27.5)	0.15
Ethnicity**			
White	25.0%	32.3%	0.39
Other	75.0%	67.6%	
Gestational age* (weeks)	34.0 (32.0 – 35.2)	34.0 (31.5 – 35.2)	0.77

BMI: Body Mass Index, pPROM: Preterm Premature Rupture of Membranes, PTL: Preterm Labor.
* Values expressed as median (min – max), and **chi-square test.

Histologic chorioamnionitis was detected in 64.5% of the amniochorion membranes from pPROM group and in 44.8% from PTL group and these frequencies were not significantly different (p=0.12). The 8-OHdG levels in the PTL group [0.71 ng/mL (min: 0.43 – max: 1.38)] were significant higher than in the pPROM group [0.53 ng/mL (min: 0.37- max: 0.69)] (p=0.02) (Figure 1). The occurence of apoptosis in the pPROM group (32.2%) was similar in the PTL group (24.1%) (p=0.48). The amniochorion membranes with histologic chorioamnionitis showed a higher apoptosis occurrence (85,1%) compared to those without chorioamnionitis (14,8%) (p=0.001) in pPROM and PTL groups. Immunolocalization of p53 protein is more prevalent in the chorionic plate (71,7%). In 28,3% both of layers membranes were stained for protein p53.

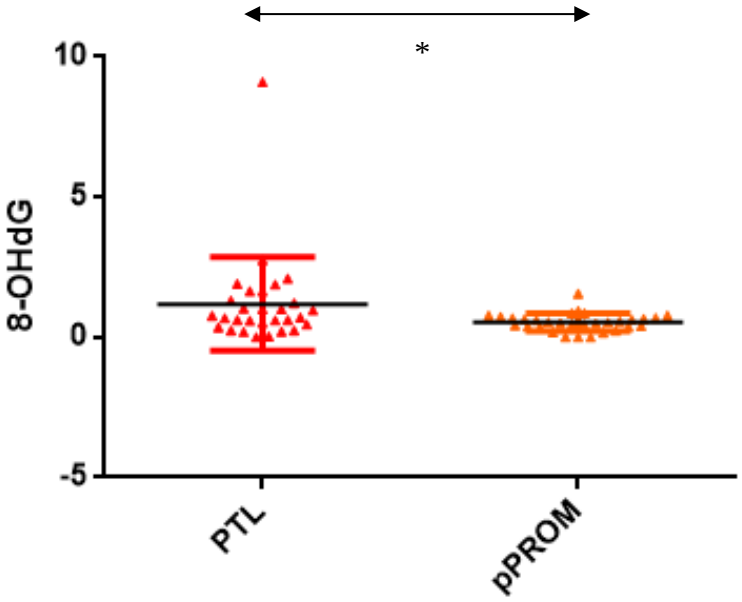
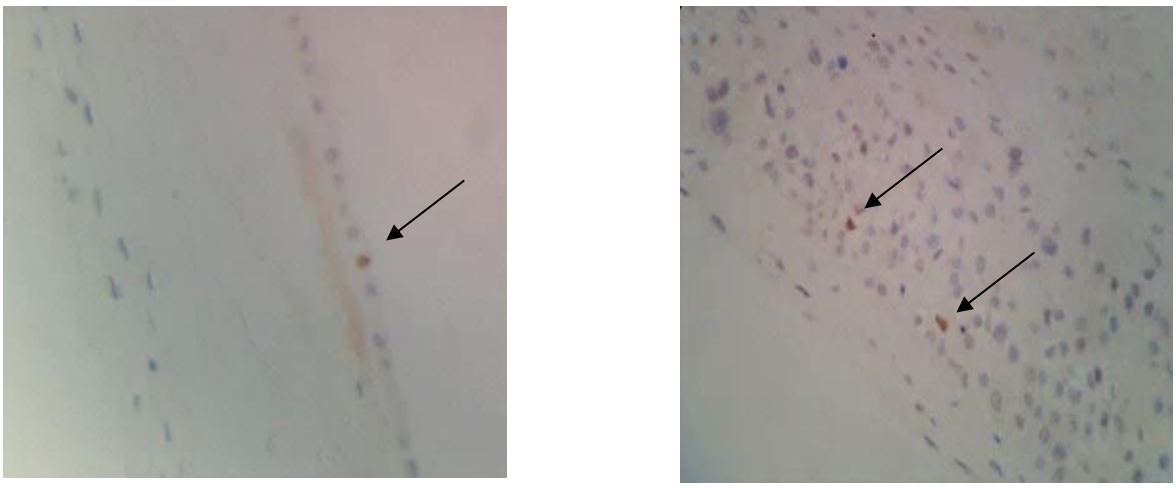


Figure 1. 8-OHdG levels in amniochorion membranes from obstetric pathologies * Mann-Whitney test.p= 0,02. Preterm labor (PTL), Premature Rupture of Preterm Ovular Membranes (pPROM).



A **B**

Figure 2. Photomicrography of the amniochorion membranes. A) p53 protein immunolocalization in the amniotic epithelium (Arrow). **B)** p53 protein immunolocalization in the chorion (Arrows)

The statistical model of multiple logistic regression used in this study indicates that the risk of apoptosis occurrence amniochorion membranes with chorioamnionitis is 3.7 times greater than in those without inflammatory infiltrate (OR=3.7; 95%CI: 1.19-14.9; p=0.04), and the other criteria, 8-OHdG (OR=0.8; 95%CI: 0.38-1.46; p=0.70), and obstetric pathologies (OR=1.1; 95%CI: 0,32-3,94;p=0,85), showed to be not related to apoptosis (Table 1).

Table 1. Associations of histologic chorioamnionitis, 8-OHdG, and obstetric pathologies with the prevalence of apoptosis by regression analysis.

Variables	Adjusted OR (95% CI)	P
Histologic chorioamnionitis	3.7 (1.19 – 14.9)	0.04
8-OHdG	0.8 (0.38 – 1.46)	0.70
Obstetric pathologies	1.1 (0.32 - 3.94)	0.85

The model showed to be adequate according to the Hosmer & Lemeshow criteria [26], showing a moderate discriminatory power estimated to be 65% (sensitivity index: 76.5%; specificity index: 53.5%) according to the ROC curve (Figure 3)

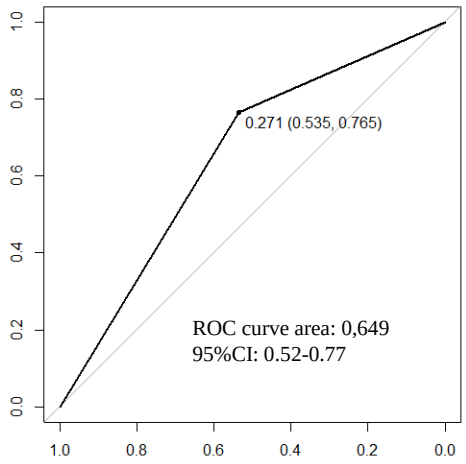


Figure 3. Area under the ROC curve for multiple logistic regression used.

DISCUSSION

This is a study on possible mechanisms involved in preterm birth taking into account that pathophysiology of preterm delivery is multifactorial, and it is not yet completely understood. The presence of histologic chorioamnionitis, oxidative stress, and apoptosis, which can alone or together, interfere with the success of human reproduction, are cited among these factors [27,28].

In our study, we observed that histologic chorioamnionitis was a frequent event, with similar behavior in pPROM and PTL groups, althoughits presence is an important element in its association with cell death diagnosis.

The presence of choriomanionitis is a well-known factor in the occurrence of adverse pregnancy outcomes. Notedly, in the preterm birth its participationis due to an exacerbated local inflammatory response [25,29]. Furthermore, activation of an inflammatory cascade is the trigger for the uterotonic signs that can induce preterm labor through intensive leukocyte activation. As a consequence, mediators of the inflammatory response and collagenases are produced, resulting in the typical loss of the contractile myometrium refractoriness and/or fragility of the amniochorion membranes [6, 30, 31, 32].

Our study showed findings similar to those of previous studies. Their authors refer that cells of the amniochorion membranes were prematurely destroyed by apoptosis in the presence of chorioamnionitis [17, 27, 33-35]. However, explanations on how the inflammatory infiltrate isassociated with apoptosis are not fully clear. Kataoka et al. (2002) also observed similar results attributing this phenomenon to the lipopolysaccharide-induced inflammatory reactions and biochemical reactions involving the Fas protein and ligand [18, 36].

Regarding 8-OHdG levels, we observed that they were higher in the group affected by PTL. Although pregnancy itself is a period of intense metabolic demand, with the release of free radicals, oxidative stress only appears when an imbalance between its production and disposal occurs. Inhibition of the redox effect is necessary and depends on the enzymatic

and non-enzymatic antioxidant components of the human organism, which are able to maintain its homeostasis [37].

Similarly, apoptosis is a physiological phenomenon, known as a programmed cell death process that occurs in various conditions, especially those triggered by an inflammatory response or cell removal after cell damage caused by genotoxic agents [38].

In vitro studies, state that DNA injuries caused by oxidative stress, especially in inflammatory processes, can activate biomolecular pathways ending in apoptosis and adverse pregnancy outcomes [39].

One of these pathways corresponds to the continued DNA repair mechanism, notably guanine nucleotide base repair after aggression caused by free radicals. Oxidative stress not controlled by antioxidant mechanisms can trigger molecular reactions such as conjugation of the 8-oxoguanine glycosylase enzyme complex to the 8-OHdG free radical, resulting in the OGG1:8-OHdG complex. This complex is characterized by activation of the guanosine triphosphate hydroxylase (Ras-GTPase) enzyme, which stimulates p53 protein transcription resulting in apoptosis of chorioamniotic cells and pPROM as a subsequent clinical outcome [39,40].

Similarly, the RAS-GTPase enzyme can evoke the production of inflammatory mediators through expression of the NF-kB protein complex, determining an inflammatory activity environment with release of uterotonic proteins and/or degradation of the ovular membrane cellular matrix. These conditions facilitate uterine contractile activity and/or pPROM, with the consequent deflagration of preterm labor [41,42].

Other pathways, such as those involving the interaction between tumor necrosis factor-α and its Fas receptor ligand (TNF-α – FasL), also determine apoptosis and may be mentioned in addition to the activator p53 protein pathway. These pathways are related to the presence of genotoxic and infectious agents and still unknown factors, allowing apoptosis to participate in the biochemical mechanisms that culminate with preterm birth [43].

The pathway involving TNF-α and FasL starts by transducing signals, with formation of other protein complexes containing cell death domains. These complexes activate a group

of proteases known as caspases (pro-enzymes found inside the cell), which are characterized by initiating apoptosis and being their effectors [6].

The pathway corresponding to p53 protein activation is initiated from DNA fragmentation, triggering its transcription, and activating the bax protein producing mechanisms. This protein becomes the responsible for the mitochondrial membrane disruption and subsequent cytochrome C release. Thus, pro-caspase 9 is converted to its active form with a subsequent cascade of effector caspases (caspases 2, 3, 6, and 7), also related to apoptosis. Similarly, an increased p53 protein expression can inhibit the production of Bcl-2, an anti-apoptotic agent responsible for the mitochondrial membrane integrity and cytochrome C contention [2].

A higher p53 protein concentration can also be related to another mechanism involving gene overexpression of metalloproteinases (MMPs), especially MMP-2, as well as blocking of metalloproteinase-inhibiting tissue factor (TIMPs). These combined biochemical events end the structural changes in gestational tissues and deflagration of term delivery, especially pPROM and PTL [16].

We conclude that histologic chorioamnionitis seems to be more clearly associated with apoptosis occurrence in amniochorion membranes from pregnancies complicated by spontaneous prematurity, possibly through the expression of pro-inflammatory mediators.

REFERENCES

1. Liu L, Johnson HL, Cousens S, et al. Child Health Epidemiology Reference Group of WHO and UNICEF. Lancet 2012; 379: 2151-2161.

2. Fortunato SJ, Menon R. Distinct molecular events suggest different pathways for preterm labor and premature rupture of membranes. Am J Obstet Gynecol 2001; 184:1399-1406.

3. Illanes SE, Nien JK, Rice GE. Preterm labor: Understanding of the Mechanism Involved to Improve Prediction and Prevention. Obstet & Gynecol Int. 2013; 2013:858506.

4. Arayici S, Simsek GK, Oncel MY, et al. The effect of histological chorioamnionitis on the short-term outcome of preterm infants <32 weeks: a single-center study. J Matern Fetal Neonatal Med 2014; 27:1129-1133.

5. Bracci R, Buonocore G. Chorioamnionitis: A Risk Factor for Fetal and Neonatal Morbidity. Biol Neonate 2003; 83:85-96.

6. Menon R, Fortunato SJ. Infection and the role of inflammation in preterm premature rupture of the membranes. Clin Obstet Gynecol 2007;21:467-478.

7. Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA: Reactive oxygen species, cell signaling, and cell injury. Free Radic Biol Med 2000;28:1456.

8. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 2012; 10:10-49.

9. Henrotin Y, Deberg M, Mathy-Hartert M, Deby-Dupont G. Biochemical biomarkers of oxidative collagen damage. Adv Clin Chem 2009; 49:31-55.

10. Sies H. Strategies of antioxidant defense. Eur J Bio 1993; 215:213-219.

11. Sies H. Oxidative stress: from basic research to clinical application. Am J Med 1991; 91: 315-85.

12. Valgimigli L, Pedulli GF, Paolini M. Measurement of oxidative stress by EPR radical-probe technique. Free Radic Biol Med 2001;31:708-716.

13. Richter C, Park JW, Ames BN. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Natl Acad Sci USA 1988; 85:6465-6467.

14. Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc Natl Acad Sci USA 1990; 87:4533-4537.

15. Subash P, Gurumurthy P,Sarasabharathi A, Cherian KM. Urunary 8-OHdG: a marker of oxidative stress to DNA and total antioxidant status in essential hypertension with South Indian population. Indian J Clin Biochem 2010; 25:127-132.

16. Fortunato SJ, Menon R, Bryant C, Lombardi SJ. Programmed cell death (apoptosis) as a possible pathway to metalloproteinase activation and fetal membrane degradation in premature rupture of membranes. Am J Obstet Gynecol 2000; 182:1468-1476.

17. Murtha AP, Auten R, Herbert WNP. Apoptosis in the chorion leave of term patients with histologic chorioamnionitis. Infect Dis Obstet Gynecol 2002; 10:93-96.

18. Kataoka S, Furuta I, Yamada H, et al. Increased Apoptosis of Human Fetal Membranes in Rupture of the Membranes and Chorioamnionitis. Placenta 2002;23:224-231.

19. Pinho MSL. Proteina p53: Algum valor clínico ou apenas pesquisa? Uma revisão da literatura. Rev Bras Coloproct 2000;20: 258-260.

20. Irwin MS, Kaelin WG. p53 family uptodate: p73 and p63 develop their own identities. Cell Growth Differ 2001; 12:337-349.

21. Vousden KH, Lu X. Live or let die: The cell's response to p53. Nature 2002; 2:594-604.

22. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 2005; 3:1-21.

23. Hung TH, Lo LM, Chiu TH Li MJ, Yeh YL, Chen SF, et al. A longitudinal study of oxidative stress and antioxidant status in women with uncomplicated pregnancies throughout gestation. Reprod Sci 2010; 17: 401-409.

24. Chai M, Barker G, Menon R, Lappas M. Increased oxidative stress in human fetal membranes overlying the cervix from term non-labouring and post labour deliveries. Placenta 2012; 33: 604-610.

25. Redline RW, Faye-Petersen O, Heller D, et al. Amniotic infection syndrome: Nosology and reproducibility of placental reaction patterns. Pediatr Dev Pathol 2003;6:435-448.

26. Hosmer DW, Lemeshow S. Applied logistic regression. Stat Med 1991; 10:1162-1163.

27. Lannon SMR, Vanderhoeven JP, Eschenbach DA, et al. Synergy and Interaction Among Biological Pathways Leading to Preterm Premature Rupture of Membranes. Reprod Sci 2014; 21:1215-1227.

28. Lima E.S., Abdalla D.S.P. Peroxidação lipídica: mecanismo e avaliação em amostras biológicas. Bra J Pharmac Sci 2001; 37:293-303.

29. Wei Shu-Qin, Fraser W, Luo Zhong-Cheng. Inflammatory Cytokines and Spontaneous Preterm Birth in Asymmtomatic Women. Obstet & Gynecol 2010; 116:393-401.

30. Kim CJ, Yoon BH, Romero R, et al. Umbilical arteritis and phlebitis mark

different stages of the fetal inflammatory response. Am J Obstet Gynecol 2001; 185:496-500.

31. Romero R, Mazor M, Munoz H, Gomez R, Galasso M, Sherer DM. The preterm labor syndrome. Ann M Y Acad Sci 1994; 734:414-429.

32. El-Shazly S, Makhseed Maʿasoumah, Azizieh F, et al. Increased Expression of Pro-Inflammatory Cytokines in Placentas of Women Undergoing Spontaneous Preterm Delivery or Premature Rupture of Membranes. Am J Reprod Immunol 2004;52:45-52.

33. Parmley TH. Spontaneous cell death in the chorion leave. Am J Obstet Gynecol 1990; 162:1576-1583.

34. George RB, Kalich J, Yonish B, et al. Apoptosis in the chorion of fetal membranes in preterm premature rupture of membranes. Am J Perinatol 2008;25:29-32.

35. Menon R, Lombardi SJ, Fortunato SJ. TNF-α Promotes Caspases Activation and Apoptosis in Human Fetal Membranes. J Assist Reprod Genet 2002;19:201-204.

36. Runic R, Lockwood CJ, LaChapelle L, et al. Apoptosis and Fas expression in human fetal membranes. J Clin Endocrinol Metab 1998; 83:660-666.

37. Gohil JT, Patel PK, Priyanka G. Evaluation of Oxidative Stress and Antioxidant Defense in Subjects of Preeclampsia. J Obstet Gynaecol India 2011; 61:638-640.

38. Fortunato SJ, Menon R, Lombardi SJ. Support for an infection-induced apoptotic pathway in human fetal membranes. Am J Obstet Gynecol 200; 184:1392-1398.

39. Menon R. Oxidative stress damage as a detrimental factor in preterm birth pathology Front Immunol 2014;5:1-13.

40. Klungland A, Bjelland S. Oxidative damage purines in DNA: role of mammalian Ogg1. DNA Repair 2007;6:481-488.

41. Khanjani S, Kandola MK, Lindstrom TM, et al. NF-kappaB regulates a cassete of immune/inflammatory genes in human pregnant myometrium at term. J Cell Mol Med 2011; 15:809-824.

42. Menon R, Polettini J, Syed TA, et al. Expression of 8-oxoguanine Glycosylase in Human fetal membranes. Am J Reprod Immunol 2014;72:75-84.

43. Athayde N, Edwin SS, Romero R, et al. A role for matrixmetalloproteinase-9 in spontaneous rupture of the fetal membranes. Am J Obstet Gynecol 1998; 179:1248-1253.

Artigo Científico III

ADVERSE NEONATAL OUTCOMES IN PREGNANCY COMPLICATED BY PRETERM PREMATURE RUPTURE OF MEMBRANES AND PRETERM LABOR RELATED TO HISTOLOGICAL CHORIOAMNIONITIS

Moisés D. de Lima[1*], Alexandre R. da Paz[2], Laura F. Martin[3], Márcia G. da Silva[3]

[1.] Department of Obstetrics and Gynecology, Health Sciences Center, Federal University of Paraíba, UFPB, João Pessoa, Brazil.

[2.] Department of Surgery, Medical Sciences Center Health Sciences Center, Federal University of Paraíba, UFPB, João Pessoa, Brazil.

[3.] Department of Pathology, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil.

*Corresponding author

Department of Obstetrics and Gynecology, Health Sciences Center,

Federal University of Paraíba, UFPB, João Pessoa, Brazil

Phone number: +55 (83) 3216-7041

E-mail: drmoiseslima@gmail.com

ABSTRACT

Introduction: Preterm birth persists globally as one of the main high-incidence obstetric complications. Histologic chorioamnionitis is found among the multiple risk factors associated with prematurity, which is characterized by the presence of polymorphonuclear cells infiltrate in the amniochorion membranes. **Objective:** To analyze the association of adverse neonatal outcomes, especially early neonatal sepsis, in pregnant women affected by Preterm Premature Rupture of Membranes (pPROM) and preterm labor (PTL) in the presence of histologic chorioamnionitis. **Methods**: It was a case-control study conducted in the Obstetrics Unit of the Lauro Wanderley University Hospital (HULW), Federal University of Paraíba (UFPB), Paraiba State, Brazil from January to December 2014 and a total of 73 pregnant women were enrolled, being 39 pregnant women who presented pPROM and 34 with PTL. After delivery, the membranes were subjected to a histopathological examination and the newborn infants were evaluated for the diagnosis of adverse events: Apgar score <7 at 5min and 10 min, admission to a neonatal intensive care unit, respiratory distress, use of continuous positive airflow pressure (CPAP), tracheal intubation and early neonatal sepsis (ENS), defined as being a clinical syndrome observed in the first 72 h of life of the newborn. **Results**: In both groups, the sample general characteristics (maternal age, body mass index, ethnicity, parity, gestational age, and weight of the newborn) were not statistically different. Except ENS, which was the most prevalent in the pPROM and chorioamnionitis groups (p=0.04), the other neonatal adverse outcomes showed no statistical difference. In a logistic regression model, we found that the presence of histologic chorioamnionitis (OR=7.4; 95%CI=1.95-35.9;p=0.0005), respiratory distress (OR=3.5; 95%CI=1.02-12.9; p=0,049) and pPROM (OR=9.8; 95%CI= 2.95-38.5; p=0.0004) among the independent variables, directly influenced ENS occurrence. ROC analysis reinforced that choriamnionites and pPROM are as criteria of relevant sensitivity (76.0%) and specificity (82.0%) for the ENS event. **Conclusion**: Histologic chorioamnionitis and occurrence of pPROM are associated with adverse neonatal outcomes, especially the occurrence of early neonatal sepsis.

Keywords: Histologic chorioamnionitis, neonatal morbidity, early neonatal sepsis.

INTRODUCTION

Prematurity constitutes one of the most important clinical intercurrences related to human reproduction and it´s defined as delivery before 37 completed weeks of gestation. Its incidence is variable, oscillating in the range of 5-18% among all pregnancies, despite the scientific advances related to the knowledge of its pathophysiology [1,2]. It occurs spontaneously in 70% of cases, spontaneous preterm birth includes preterm delivery preceded by preterm labor with intact membranes (PTL) and preterm premature rupture of membranes (pPROM)) [3].

Inflammatory mechanisms are among those most associated with prematurity, and special attention has been given to amniochorion membranes, as they are a commonly involved tissue, being able to trigger immune-inflammatory responses with local and systemic effects [4, 5].

Chorioamnionitis or intra-amniotic infection designates the state in which this inflammatory process is evidenced, being characterized by impairment of the amniochorion membranes, amniotic fluid, placenta, and/or maternal decidua [6], being yeta risk factor probably related to the maternal and neonatal adverse outcomes [7].

The most common cause of amniotic cavity contamination comes from biological agents that rise from the lower female genital tract [8,9]. The hematogenic pathway and microorganism migration from the abdomen into the uterus through the uterine tubes or as a result of obstetrical procedures, such as amniocentesis or chorionic villus biopsy, are other less common forms of contamination [10,11]. Other apparently-sterile pathological conditions of the uterine cavity are also observed, being probably an innate inflammatory response of constitutional character [12].

In its clinical form, chorioamnionitis is observed in term births (1-2%) and preterm births (5-10%) [13].The prevalence is variable in its histological form, seeming intrinsically related to gestational age with rates that vary in the range 5-94% [14].

Among the microorganisms most found in the uterine cavity of pregnant women with intra-amniotic infection are as follows: *Ureaplasma urealitycum* and *Mycoplasma hominis*, which are *Fusobacterium* species; occasionally, Gram-negative bacilli, such as those of the *Bacteroides* and *Prevotela* genera, both present in bacterial vaginosis, are also found coliforms such as *Escherichia coli* and group-B *Streptococcus*. In this scenario, we highlight the pathological core of bacterial vaginosis, which installs itself in the fetal membranes and invades the amniotic cavity, proliferating in the amniotic fluid [15-17].

Once infected, the amniotic cavity induces an exaggerated inflammatory response mediated by the increase in the levels of pro-inflammatory cytokines, metalloproteinases, and prostaglandins. They favor the events that culminate with delivery deflagration, which generally is premature [18-21]. Its presence also seems related to an amplification of immediate damage to the neonate, especially early neonatal sepsis (ENS), possibly due to their inflammatory and/or infectious environment that occurs from the uterine cavity. In the long term, sequels such as motor, visual, and cognitive disabilities is also reported in this condition [22-24].

The purpose of this study is to examine obstetric complications involving prematurity and analyze the association between adverse neonatal outcomes, especially, ENS, related to the presence of histologic chorioamnionitis.

MATERIAL AND METHODS

It was a case-control study conducted in the Obstetrics Unit of the Lauro Wanderley University Hospital (HULW), Federal University of Paraíba (UFPB), Paraiba State, Brazil from January to December 2014 and a total of 73 pregnant women were enrolled, being 39 pregnant women who presented pPROM and 34 with PTL. The groups were between 23 and 36 gestational weeks confirmed by both date of last menstruation and obstetric echography performed up to the 12 week of pregnancy.

Diagnosis of pPROM was considered when spontaneous loss of amniotic fluid occurred, as visualized by physical examination. This exam was performed using a sterile vaginal speculum before the patients have completed 37 weeks of gestation, and confirmed by the Amni Sure test. The presence of regular uterine contractions (3/10 min) and cervical dilation (≥3 cm) [3] were the definition of PTL.

Women with preeclampsia, HELLP syndrome, placenta previa, placental abruption, restriction in intrauterine growth, cervical incompetence, oligohydramnios, polyhydramnios, fetal malformation, stillbirth, HIV-AIDS, gestational diabetes, Rh incompatibility, and illicit drug users were excluded.

Information on maternal age, body mass index (BMI; as defined by the World Health Organization, WHO), ethnicity (self-reported), parity, gestational age, and newborn weight were obtained in the medical records to compose the general characteristics in the groups analyzed.

The neonatal morbidity criteria were defined as APGAR score <7 at 5 min and 10 min of life, admission to the neonatal intensive care unit (ICU-Neo) for at least 7 days, and early neonatal sepsis, which was established as being a clinical syndrome observed in the first 72h of the newborn's life. To diagnose it, we have defined it as being a clinic syndrome considering the following criteria: maternal risk factor (pPROM or PTL diagnosis), presence of respiratory distress (tachypnea, moaning, chest retractions, nasal wing beats, with persistent need for oxygen supplementation and the need for continuous positive airway pressureand/or tracheal intubation for at least one day), and presence of laboratory abnormalities (elevation in blood levels of C-reactive protein (CRP), ≥ 6 mg/L, and immature neutrophils / total neutrophils ratio, (I/T), ≥ 2 [25].

After birth, the placenta and amniochorion membranes were collected in sterile conditions, and fragments near the rupture region of fetal membranes were sectioned and fixed in 10% formalin. Then, the samples were subjected to histopathological analysis by using the hematoxylin-eosin preparation technique, and the diagnosis was performed by a single examiner, without prior knowledge of the clinical conditions related to thatcase.

The histopathologic criteria used for chorioamnionitis diagnosis was that one established by Redline et al.[26] considering the presence of maternal inflammatory response in the chorioamniotic membranes in the following conditions: acute subchorionitis or early acute chorionitis, characterized by the presence of neutrophils in the subchorionic and/or chorionic spaces (**stage 1**); acute chorioamnionitis characterized by neutrophil infiltration in the chorionic connective tissue and/or chorionic plate (**stage 2**), and necrotizing chorioamnionitis, a necrosis that reached the amnion (**stage 3**). Regarding degree, the criterion establishesthatthe group of maternal neutrophils, which are diffusely in the chorionic plate, chorion, or amnion, as being mild to moderate (**grade 1**) and refers to the presence of at least three microabscesses as severe (**grade 2**) Redline et al.[26].

During the hospitalization period, all women participating in the study were assisted according to the clinical protocols of medical service at the HULW-UFPB. The research projectwas approved by the Ethics Committee Board in Research of UFPB (Protocol: 1.806.905) and written informed consent was obtained from all the participants.

Statistical analyzes were performed by using the SigmaStat 3.5Software (Copyright©2006 Systat Software, Inc. San Jose, CA, USA). Continuous variables were presented as medians and minimum and maximum values and analyzed by using the Mann-Whitney U-test. Categorical variables were analyzed by the Fisher's exact or chi-squared tests, being presented as proportions when necessary. A logistic regression model, which was performed by using the public-domain R (Free Software Foundation's GNU General Public License) software, was used to establish the adjusted ENS odds ratio for each preset control variable (chorioamnionitis, gestational age ≤ 34.0 weeks, respiratory distress, CRP value ≥ 6 mg/L, I/T ratio ≥ 2, and obstetrical pathology). The p value <0.05 were considered statistically significant.

RESULTS

General maternal and neonatal characteristics of the patients included in the study in pPROM and PTL groups are presented in Table 1. Similar results were observed for maternal age, body mass index (BMI), ethnicity, parity, gestational age and weight of the newborn.

Table 1. General maternal and neonatal characteristics of the patients included in the study groups.

	pPROM n = 39	PTL n = 34	*p*
Maternal age* (years)	25 (19.2 – 32.0)	23.5 (19.0 – 27.0)	0.261
BMI*	26.4 (23.8 - 29.3)	25.2 (23.4 – 27.5)	0.155
Ethnicity			
Mulatto£	27 (69.2%)	21 (61.7%)	0.50
Black**	02 (5.1%)	02 (5.8%)	1.00
White£	10 (25.6%)	11 (32.3%)	0.52
Parity			
< 3	37 (94.8%)	28 (82.3%)	
> 3	02 (5.2%)	06 (17.7%)	0.13**
Gestational age* (weeks)	34.0 (32.1-35.3)	33.8 (31.4-35.2)	0.69
Newborn weight (grams)*	2335 (1780-2656)	2270 (1655-2595)	0.69

BMI: Body Mass Index, pPROM: Preterm Premature Rupture of Membranes, PTL: Preterm Labor.
* Values expressed as median (min - max), £ Fisher's exact test and **chi-square test.

The diagnostic of histological chorioamnionitis was present in 64.1% (25/39) of the patients with pPROM and in 50.0% (17/34) in PTL group and no significant difference was found in the chorioamnionitis frequency in theboth groups (p=0.22).

The frequency of the histologic chorioamnionitis was estimated considering the gestational age at delivery. The data obtained in this study reflect that the proportion was not statistically different in the groups 23-34 weeks (32.4%) and 34-36 weeks (25.0%) (p=0.48).

The adverse neonatal outcomes were analyzed in each group and between the groups pPROM and PTL related to the presence/absence of histological chorioamnionitis and the data are exposed in Table 2.

Table 2. Adverse neonatal outcomes in pPROM and PTL groups related to histologic chorioamnionitis presence.

	pPROM (n = 39)		*p*	PTL (n = 34)		*p*	*p* between groups
	Presence of chorioamnionitis n = 25	Absence of chorioamnionitis n = 14		Presence of chorioamnionitis n = 17	Absence of chorioamnionitis n = 17		
Apgar 5 min							
< 7	10(40.0%)	4(28.5%)	0.47	8(47.0%)	3(21.4%)	0.06	1.00
> 7	15(60.0%)	10(71.4%)		9(52.9%)	14(82.3%)		0.14
Apgar 10 min							
< 7	2(8.0%)	0 (0.0%)	0.52	4(23.5%)	2(11.7%)	0.65	1.00
> 7	23(92.0%)	14(100.0%)		13(76.4%)	15(88.2%)		0.20
Respiratory distress	15(60.0%)	7(50.0%)	0.54	6(35.1%)	7(41.1%)	0.72	0.19
Admission to ICU-Neo ≥ 7 days	17(68.0%)	11(78.5%)	0.71	6(35.2%)	3(17.6%)	0.43	1.00
CPAP use ≥1 day	15(60.0%)	7(50.0%)	0.54	6(35.2%)	7(41.1%)	0.72	0.19
Tracheal intubation ≥1 day	14(56.0%)	9(64.2%)	0.61	9(53.0%)	6(35.2%)	0.30	0.95
Early neonatal sepsis	10(40.0%)	3(21.4%)	0.04	4(23.5%)	2(11.7%)	0.27	0.99
CRP > 6 mg/L	15(60.0%)	4(28.5%)	0.04	11(64.7%)	5(29.4%)	0.03	0.70
I/T> 2	10(40.0%)	1(7.1%)	0.03	8(47.0%)	2(11.7%)	0.02	0.58
Early neonatal mortality	-	-		1(5.9%)	-	-	-

CPAP: Continuous positive airway pressure; CRP: C-reactive protein; I/T: Rate of immature/total neutrophils, pPROM: Preterm Premature Rupture of Membranes, PTL: Preterm Labor.The *p* values for categorical variables were calculated by the chi-square and Fisher's exact tests when appropriated.

The APGAR indices at 5º and 10º minutes, presence of respiratory distress, need for admission in the ICU-Neo, use ofcontinuous positive airway pressure and tracheal intubation did not differ in the groups and among the analyzed groups, considering the histologic chorioamnionitis status.

A higher prevalence of children affected by early neonatal sepsis was observed in the pPROM group in association with histologic chorioamnionitis (p=0.04) whereas a significant difference was not observed in the PTL group (p=0.27), and such prevalence was also not different between groups (p=0.99) considering the presence of inflammatory infiltrate. It was also demonstrated that levels of CRP > 6 mg/L in the pPROM and PTL groups associated with histologic chorioamnionitis are different from those not associated with chorioamnionitis, although the difference between presence or absence of chorioamnionitis between the pPROM and PTL groups is not statistically significant (p=0.70). Similarly, the rates of immature and total neutrophils (I/T) > 2, are different in the pPROM and and PTL groups associated (p=0.03) and not associated (p=0.02) with chorioamnionitis, although the difference between presence or absence of chorioamnionitis between the pPROM and PTL groups is not statistically significant (p=0.58).

The odds ratios estimated for the occurrence of early neonatal sepsis were assessed a function of variables: histologic chorioamnionitis, gestational age, respiratory distress, C-reactive protein, rate of immature/total neutrophils and obstetrical pathology. Histologic chorioamnionitis (p=0.005), respiratory distress (p=0.049) and pPROM (p=0.0004) confer significant probabilities to the development of an ENS compared to the other variables, demonstrating an excellent discriminatory power as a whole, with sensitivity around (76.0%) and specificity (82.0%) levels as exposed in the ROC curve (Table 3, Figure 3).

Table 3. Associations of histologic chorioamnionitis, respiratory distress, pPROM with the prevalence of early neonatal sepsis by regression analysis.

Variables	Adjusted OR (95% CI)	P
Histologic chorioamnionitis	7.475 (1.956 – 35.932)	0.0058
Respiratory distress	3.491 (1.026 - 12.928)	0.0497
pPROM	9.876 (2.958 – 38.519)	0.0004

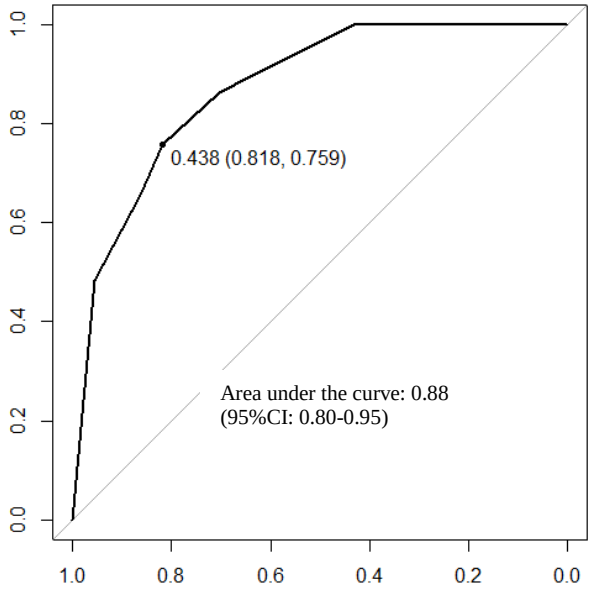


Figure 3. Area under ROC curve for predictive value of histologic chorioamnionitis, respiratory distress, Preterm Premature Rupture of Membranes for early neonatal sepsis.

DISCUSSION

Preterm birth persists as a major medical and social problem, responsible for several short and long-term morbidities during infancy [1]. The lack of better-accurate predictive methods has contributed to maintain high rates of premature births, largely because of bothmultiple factors involved in its pathophysiology and the intriguing fact that spontaneous preterm birth in women without clinical and/or gestational risk factors is the most commonly observed phenotype [27].

Chorioamnionitis is one of the most well known risk factors related to prematurity [28,29]. In this regard, emphasizing the role of amniochorion membranes is important, because they behave as a tissue capable of initiating inflammatory phenomena, especially those arising from the presence of biological pathogens such as bacteria. They cross the amniochorion membranes, reach the uterine cavity, install themselves in the gestational tissues, giving rise to an environment of subclinical septic [30, 31, 10].

The phenomena that follow chorioamnionitis installation are described as higher concentration of pro-inflammatory cytokines [32-35], significant gene expression of matrix metalloproteinases, which degrade the ovular membrane protein matrix and result in their fragility (as observed in the pPROM cases), and increased production of prostaglandins that insidiously change the cervix anatomy and activate myometrial contractions in PTL[36-38].

In recent studies, damage to the fetus and newborn has been attributed to these mediators, in addition to their deleterious effects on the pregnancy course. The risk of early neonatal sepsis, chronic lung disease, necrotizing enterocolitis, intraventricular hemorrhage, periventricular leukomalacia, cerebral palsy, and neuromotor development failure are among the perinatal adverse outcomes associated with chorioamnionitis [39]. The presence of pro-inflammatory agents, which are produced in the maternal-fetal compartment and determine systemic injuries initiated since the intrauterine life, is added to these damage, which result from the prematurity-related immaturity of fetal tissue [40-43].

Early neonatal sepsis is a clinical syndrome related to maternal risk factors such as pPROM (>18h), clinical and/or histologic chorioamnionitis, maternal fever (>37.5 ^{o}C), colonization by *Streptococcus agalactiae*, and urinary tract infection at birth, being defined as a set of clinical signs of neonatal impairment and laboratory test changes in the first 72 h of life [25].

The confirmed ENS rate vary in the range of 0.01-0.52 per 1000 live births, and about 395,000 newborns is treated as clinical conditions suspected of ENS[44]. As the supplementary laboratory tests demonstrate conflicting results, the presence of clinical signs and symptoms remain as elements of great importance in the diagnosis of neonatal sepsis. Blood culture is a laboratory test that has shown low sensitivity and with a high number of false-positive results, although it is considered a gold standard examination [45]. Tracheal aspirateculture can be a useful examination in the first 8 h of life to identify cases of congenital pneumonia [46]. The overall leukocyte count showsto be unreliable in the first hours of life due to the variability in leukopoiesis dynamics, although a more specific analysis of the blood white series can significantly contribute to the diagnosis. Neutropenia, for example, seems to be a better predictor of early neonatal sepsis, as it demonstrates the rapid depletion of marrow reserve. Similarly, the relationship between immature and total neutrophils ratio proves to be a better predictor of sepsis when their values are equal to or greater than 0.2 [47]. The C-reactive protein is a rapid-phase marker of inflammatory activity and its serial rise in the first 24-48 h has been believed to provide a diagnostic sensitivity for neonatal sepsis of about 92% [48-50].

The data obtained in the present study showed to be consistent with those of the literature that correlate chorioamnionitis with premature birth, especially those that occur in women affected by pPROM [51,52], thus confirming previous studies that emphasize the role of acute inflammation withpregnancy interruption by the second gestational trimester [53,54]. Likewise, we have observed that the isolated criteria established as chorioamnionitis related adverse neonatal outcomes, do not seem to be different between the groups affected by

pPROM, PTL, or even between them, except for the evidence of a higher prevalence of early sepsis in the pPROM and choriomnionitis groups.

Taking into account the neonatal outcome of early sepsis, our results allow us to infer that the presence of inflammatory infiltrate, respiratory distress and pPROM representthe conditions most related to the objectiveof this study. They resulted in an almost tenfold chance of developing neonatal sepsis and showed to be as criteria variables of important sensitivity and specificity in relation to the outcome.

In this sense, our results were similar to those reported in the study by Dexter et al. [55] wherethe chances of early neonatal sepsis in low-weight newborns and chorioamnionitis had about five times higher. In a case-control study, Arayici et al.[6] refer a probability of about 40% for infants with less than 32 gestation weeks to develop neonatal sepsis. Similarly, Ahn et al. [56] found an odds ratio of about nine times more for early neonatal sepsis when chorioamnionitis was present, and Bersani et al. [57] reported a higher chorioamnionitis contribution to the early neonatal sepsis rates. Interestingly, these authors reported a protective effect of inflammation on the respiratory stress syndrome and of late neonatal sepsis in neonates.

Despite the limitations of this study, which include the sample size, diversity of diagnostic criteria for histologic chorioamnionitis and early neonatal sepsis, it was possible to conclude that the early neonatal sepse of the newborn is directly related to the presence of histologic chorioamnionitis, respiratory distress and pPROM. Besides that, the reduction in the number of adverse neonatal outcomes, including prematurity and its consequences, still need a better understanding of the pathophysiological mechanisms and development of predictive methods with higher accuracy.

REFERENCES

1. Liu L,Johnson HL, Cousens S, et al. Child Health Epidemiology Reference Group of WHO and UNICEF. Lancet 2012; 379: 2151-2161.

2. Blencowe H, Cousens S, Oestergaard MZ et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications.Lancet. 2012; 379: 2162-2172.

3. Corrêa MD, Júnior MDC. Parto Pretermo. In: Rezende J. Obstetrícia. 9.ed. Rio de Janeiro: Guanabara Koogan; 2002; p.892-914.

4. Menon R. Oxidative stress damage as a detrimental factor in preterm birth pathology. Front Immunol 2014;5:1-13.

5. Than NG, Kim SS, Abbas A, et al.Chorioamnionitis and Increased Galectin-1 Expression in PPROM – An Anti-Inflammatory Response in the Fetal Membranes? Am J Reprod Immunol 2008;60:298-311.

6. Arayici S, Kadioglu Simsek G, Oncel MY, et al. The effect of histological chorioamnionitis on the short-term outcome of preterm infants <32 weeks: a single-center study. J Matern Fetal Neonatal Med 2014;27:1129-1133

7. Bracci R, Buonocore G. Chorioamnionitis: A Risk Factor for Fetal and Neonatal Morbidity. Biol Neonate 2003; 83:85-96.

8. Galask RP, Varner MW, Petzold CR, Wilbur SLl. Bacterial attachment to the chorioamniotic membranes. Am J Obstet Gynecol 1984;148:915-28.

9. Romero R, Mazor M. Infection and preterm labor. Clin Obstet Gynecol 1988; 31:553-84.

10. Redline RW. Placental inflammation. Semin Neonatol 2004; 9:265-274.

11. Romero R, Miranda J, Chaiworapongsa T, et al. A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. Am J Reprod Immunol 2014; 71:330-358.

12. Romero R, Miranda J, Chaemsaithong P, et al. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med 2014; 29:1-16.

13. Gibbs RS, Duff P. Progress in phathogenesis and management of clinical intraamniotic infection. Am J Obstet Gynecol 1991;164:1317-1326.

14. Kim CJ, Romero R, Chaemsaithong P, et al. Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. Am J Obstet Gynecol 2015; 214:S29-S52.

15. Sperling RS, Newton E, Gibbs RS. Intra-amniotic infection in low-birth-weight infants. J Infect Dis 1988; 157:113-117.

16. Romero R, Mazor M, Munoz H, et al. The preterm labor syndrome. Ann M Y Acad Sci 1994;734:414-429.

17. Gibbs RS, Blanco JD, St Clair PJ, et al. Quantitative bacteriology of amniotic fluid from women with clinical intraamniotic infection at term. J Infect Dis 1982;145:1-8.

18. Romero R, Brody DT, Oyarzun E, et al. Infection and labor: III, interleukin-1: a signal for the onset of parturition. Am J Obstet Gynecol 1989; 160:1117-1123.

19. Romero R, Mazor M, Brandt F, et al. Interleukin-1 alpha and interlukin-1 beta in preterm and term human parturition. Am J Reprod Immunol 1992; 27:117-123.

20. Romero R, Avila C, Santhanam U, et al. Amniotic fluid interleukin-6 in preterm labor: association with infection. J Clin Invest 1990;85:1392-1400.

21. Figueroa R, Garry D, Elimian A, et al. Evaluation of amniotic fluid cytokines in preterm labor and intact membranes. J Matern Fetal Neonatal Med 2005;18:241-247.

22. Ericson JE, Laughon MM. Chorioamnionitis implications for the neonate. Clin Perinatol 2015; 42:155-165.

23. Avchen RN, Scott KG, Mason CA. Birth weight and school-age disabilities: a population-based study. Am J Epidemiol 2001;154: 895–901.

24. Swamy GK, Ostbye T, Skjaerven R. Association of preterm birth with long-term survival, reproduction, and next-generation preterm birth. JAMA 2008; 299:1429–1436.

25. Silveira RC, Procianoy RS. Uma revisão atual sobre sepse neonatal. Bol Cient Pediatr 2012; 1:29-35.

26. Redline RW, Faye-Petersen O, Heller D, et al. Amniotic infection syndrome: Nosology and reproducibility of placental reaction patterns. Pediatr Dev Pathol 2003; 6:435-448.

27. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet 2008; 371:75-84.

28. Erdemir G, Kultursay N, Calkavur S, et al. Histological chorioamnionitis: effects on premature delivery and neonatal prognosis. Pediatr Neonatol 2013; 54:267-274.

29. Guzick DS, Winn K. The association of chorioamnionitis with preterm deflivery. Obstet Gynecol 1985; 65:11-16.

30. Galask RP, Varner MW, Petzold CR, et al. Bacterial attachment to the chorioamniotic membranes. Am J Obstet Gynecol 1984;148:915-928.

31. Romero R, Gomez R, Chaiworapongsa T, et al. The role of infection in preterm labour and delivery. Paediatr Perinat Epidemiol 2001; 15:41-56.

32. Hillier SL, Witkin SS, Krohn MA, et al. The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamniotic and chorioamnion infection. Obstet Gynecol 1993; 81:941-948.

33. Yoon BH, Romero R, Jun JK, et al. Amniotic fluid cytokines (interlukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. Am J Obstet Gynecol 1997; 177:825-830.

34. Romero R, Mazor M, Sepulveda W, et al. Tumor necrosis factor in preterm and term labor. Am J Obstet Gynecol 1992; 166:1576-1587.

35. Romero R, Sepulveda W, Kenney JS, et al. Interleukin-6 determination in the detection of microbial invasion of the amniotic cavity. Ciba Found Symp 1992;167:205-223.

36. Athayde N, Edwin SS, Romero R, et al. A role for matrix metalloproteinase-9 in spontaneous rupture of the fetal membranes. Am J Obstet Gynecol 1998;179:1248-1253.

37. Maymon E, Romero R, Pacora P, et al. Human neutrophil collagenase (matrix metalloproteinase 8) in parturition, premature rupture of the membranes, and intrauterine infection. Am J Obstet Gynecol 2000; 183:94-99.

38. Moon JB, Kim JC, Yoon BH, et al. Amniotic fluid matrix metalloproteinase-8 and the development of cerebral palsy. J Perinat Med 2002;30:301-306.

39. Perrone S, Toti P, Toti MS, et al. Perinatal outcome and placental histological characteristics: a single-center study. J Matern Fetal Neonatal Med 2012; 25:110-113.

40. Schmidt B, Cao L, Mackensen-Haen S, et al. Chorioamnionitis and inflammation of the fetal lung. Am J Obstet Gynecol 2001; 184:173-177.

41. De Dooy JJ, Mahieu LM, van Bever HP. The role of inflammation in the development of chronic lung disease in neonates. Eur J Pediatr 2001;160:457-463.

42. Gilstrap LC, Ramin SM. Infection and cerebral palsy. Semin Perinatol 2000; 24:200-203.

43. Redline RW, O'Riordan MA. Placental lesions associated with cerebral palsy and neurologic impairment following term birth. Arch Pathol Lab Med 2000;124:1785-1791.

44. van Herk W, Stocker M, van Rossum Am. Recognising early onset neonatal sepsis: an essential step in appropriate antimicrobial use. J Infect 2016; 5:S77-S82.

45. Thuler LC. Diagnóstico microbiológico das bacteremias. JBM 1995;69:123-128.

46. Sherman MP, Goetzman BW, Ahlfors CE. Tracheal aspiration and its clinical correlates in the diagnosis of congenital pneumonia. Pediatrics 1980; 65:258-262.

47. Rodwell RL, Taylor KM, Tudehope DI, et al. Early diagnosis of neonatal sepsis using a hematologic scoring system. J Pediatr 1988;112:761-767.

48. Mathers NJ, Polhandt F. Diagnostic audit of C-reactive protein in neonatal infection. Eur J Pediatr 1987;146:147-151.

49. Lee Sy, Park KH, Jeong EH, et al. Relationship between maternal serum C-reactive protein, funisitis and early-onset neonatal sepsis. J Korean Med Sci 2012; 27:674-680.

50. Hofer N, Zacharias E, Muller W, et al. An Update on the Use of C-Reactive Protein in Early-Onset Neonatal Sepsis: Current Insights and New Tasks. Neonatology 2012;102:25-36.

51. Park HS, Romero R, Lee SM, et al. Histologic chorioamnionitis is more common after spontaneous labor than after induced labor at term. Placenta 2010;31:792-795.

52. Lee SM, Lee KA, Kim SM, et al. The risk of intra-amniotic infection, inflammation and histologic chorioamonionitis in term pregnant women with intact membranes and labor. Placenta 2011;32:516-521.

53. Lee SM, Park JW, Kim BJ, et al. Acute histologic chorioamnionitis is an risk factor for adverse neonatal outcome in late preterm birth after preterm premature rupture of membranes. PLoS One 2013; 8:e79941.

54. Thomas W, Speer CP. Chorioamnionitis: important risk factor or innocent bystander for neonatal outcome? Neonatology 2011; 99:177-187.

55. Dexter SC, Malee MP, Pinar H, et al. Influence of chorioamnionitis on developmental outcome in very low birth weight infants. Obstet Gynecol 1999; 9:267-273.

56. Ahn HM, Park EA, Cho SJ, et al. The association of histological chorioamnionitis and antenatal steroids on neonatal outcome in preterm infants born at less than thirty-four weeks gestation. Neonatology 2012;102:259-264.

57. Bersani I, Thomas W, Speer CP. Chorioamnionitis – the good or the evil for neonatal outcome? Matern Fetal Neonatal Med 2012;25:12-16.

Conclusões

Considerando o tamanho amostral incluído nesse estudo bem como as metodologias empregadas para atingir os objetivos propostos, podemos concluir que:

1) Nossos dados reforçam que os danos oxidativos estão presentes em gestações a termo como processo fisiológico do envelhecimento das membranas corioamnióticas;
2) A corioamnionite histológica, entre os mecanismos analisados, parece estar mais claramente associada à ocorrência de apoptose em membranas corioamnióticas de gestações complicadas pela prematuridade espontânea.
3) A Rotura Prematura de Membras Pré-Termo e a corioamnionite histológica estão associadas a desfechos neonatais adversos, especialmente à ocorrência de sepse neonatal precoce.

Anexos

HOSPITAL UNIVERSITÁRIO
LAURO WANDERLEY/UFPB

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Análise dos mecanismos fisiopatológicos relacionados ao parto pré-termo: corioamnionite histológica, estresse oxidativo e apoptose

Pesquisador: Moisés Diogo de Lima

Área Temática: A critério do CEP

Versão: 4

CAAE: 19474713.8.0000.5183

Instituição Proponente: Hospital Universitário Lauro Wanderley/UFPB

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 1.806.905

Apresentação do Projeto:

Trata-se de uma Emenda ao protocolo de pesquisa, que tem como pesquisador responsável Dr. Moisés Diogo de Lima, aprovado por este CEP - Número do Parecer: 1.255.858.

A emenda foi solicitada devido sugestão de alteração do título da pesquisa pela orientadora, não havendo quaisquer outras alterações no conteúdo metodológico do estudo.

Título primário: Análise de mediadores pró e anti-inflamatórios e de marcadores de estresse oxidativo em membranas corioamnióticas de gestações complicadas por Rotura Prematura de Membranas Pré-Termo e Trabalho de Parto

Título proposto: Análise dos mecanismos fisiopatológicos relacionados ao parto pré-termo: corioamnionite histológica, estresse oxidativo e apoptose

Objetivo da Pesquisa:

OBJETIVO PRIMÁRIO

O objetivo do presente estudo é avaliar a expressão de mediadores pró e anti-inflamatórios (IL-1, IL-6, IL-8, IL-10, IL-18, IL-33, ST2, TNF-alfa, PTX- 3, Galectina-1, TLR-1, TLR-2, TLR-4, TLR-6, Ativina A, Receptor de Ativina, NOD-1 e NOD-2) e de marcadores de estresse oxidativo (8-IsoP,

Endereço: Hospital Universitário Lauro Wanderley - 2º andar - Campus I - UFPB.
Bairro: Cidade Universitária **CEP:** 58.059-900
UF: PB **Município:** JOAO PESSOA
Telefone: (83)3216-7964 **Fax:** (83)3216-7522 **E-mail:** comitedeetica@hulw.ufpb.br

MDA, 3-NT e 8-OHdG) em membranas corioamnióticas de gestações de termo e complicadas por TPP e RPM-PT que culminarem em
parto pré-termo, bem como caracterizar os perfis desses marcadores nestas diferentes manifestações clínicas da prematuridade.

Avaliação dos Riscos e Benefícios:

Riscos e benefícios avaliados e declarados adequadamente, em consonância com a Resolução 466/2012, do CNS/MS.

Comentários e Considerações sobre a Pesquisa:

Considerando a manutenção dos aspectos éticos e metodológicos do estudo, conforme aprovação por este Colegiado anteriormente, não há qualquer impedimento para alteração do título proposto, uma vez que não haverá mudanças no protocolo de pesquisa.

Considerações sobre os Termos de apresentação obrigatória:

Foram apresentados:
Documento solicitando mudança de título da pesquisa emitida pelo pesquisador responsável, com anuência da Orientadora.

Recomendações:

Recomendamos que ao término do estudo seja enviado ao CEP/HULW, o relatório final para emissão da certidão definitiva.

Conclusões ou Pendências e Lista de Inadequações:

Diante da solicitação, em atendimento às diretrizes éticas da Resolução 466/2012, somos de parecer favorável a mudança do título do estudo.

Considerações Finais a critério do CEP:

Ratificamos o parecer de APROVAÇÃO da Emenda, emitido pelo Colegiado do CEP/HULW, em 'ad referendum'.

O presente projeto, seguiu nesta data para análise da CONEP e só tem o seu início autorizado após a aprovação pela mesma.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_820993_E1.pdf	04/11/2016 13:16:32		Aceito
Outros	TITULO_MOISES_DEFESA.pdf	04/11/2016 13:14:30	Moisés Diogo de Lima	Aceito
Folha de Rosto	folha_rosto_moises.pdf	04/11/2016	Moisés Diogo de	Aceito

Folha de Rosto	folha_rosto_moises.pdf	13:10:05	Lima	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TERMODECONSENTIMENTO.doc	23/09/2015 16:37:48	Moisés Diogo de Lima	Aceito
Projeto Detalhado / Brochura Investigador	Projeto Pesquisa.docx	24/03/2015 14:30:21		Aceito
Outros	Carta CONEP JP 2015.docx	24/03/2015 10:39:10		Aceito
Outros	Carta de Anuência ICV 2015.pdf	24/03/2015 10:35:17		Aceito
Outros	CEGEP 2015.pdf	24/03/2015 10:35:04		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Sim

JOAO PESSOA, 06 de Novembro de 2016

Assinado por:
MARIA ELIANE MOREIRA FREIRE
(Coordenador)