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ANÁLISE DAS INTERLEUCINAS 6, 8,10 E 17 EM NEONATOS PREMATUROS COM DISPLASIA BRONCOPULMONAR

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ANÁLISE DAS INTERLEUCINAS 6, 8,10 E 17 EM NEONATOS PREMATUROS COM DISPLASIA BRONCOPULMONAR

Tese de doutorado apresentada ao Curso de Pós Graduação em Ciências da Saúde (PPGCS) da Pontifícia Universidade Católica do Paraná (PUCPR), como requisito para obtenção do título de Doutora.

Orientadora: Professora Doutora Lucia de Noronha

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DEDICATÓRIA

Aos meus pais, marido e filhas dedico todo o meu amor e trabalho

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RESUMO

A displasia broncopulmonar (DBP) é o resultado da alteração do desenvolvimento pulmonar do neonato prematuro. A fisiopatologia é incerta e a resposta inflamatória à lesão pulmonar pode estar envolvida neste processo. **Objetivo:** Avaliar o papel das interleucinas 6, 8, 10 e 17 na DBP por meio de estudo anatomopatológico e imuno-histoquímico dos pulmões de neonatos prematuros com DBP. **Método:** Foram selecionados 32 casos de necropsias neonatais do Departamento de Patologia do Hospital de Clínicas da Universidade Federal do Paraná, realizados entre 1991 e 2005. A amostra incluiu neonatos com menos de 34 semanas de idade gestacional submetidos à oxigenoterapia e com amostras pulmonares fixadas em formalina e conservadas em parafina. Os espécimes pulmonares foram posteriormente classificados em 3 grupos de acordo com as alterações histopatológicas e morfométricas (DBP clássica, DBP nova e sem DBP), sendo submetidos à análise imuno-histoquímica. Os anticorpos selecionados para o estudo foram anticorpos monoclonais anti-IL-6, IL-8, IL-10 e IL-17. **Resultados:** As interleucinas IL-6, IL-8 e IL-10 não apresentaram diferenças significativas na imunoexpressão tecidual entre os grupos. A IL-17 apresentou maior imunorreatividade tecidual no grupo sem DBP em comparação ao grupo com DBP clássica (1686 vs 866 μm2, p = 0,029). **Conclusão:** Este estudo com tecido pulmonar de neonatos prematuros com DBP mostrou que as ILs-6, 8, 10 e 17 podem não estar envolvidas na fisiopatologia da DBP.

Palavras Chaves: displasia broncopulmonar, interleucina, neonato, inflamação, pulmão, biomarcador

ABSTRACT

Bronchopulmonary dysplasia (BPD) is an abnormality in premature neonatal lung development. The pathophysiology is uncertain and the inflammatory response to lung injury may be the pathway. **Objective:** To evaluate the role of interleukins 6, 8, 10 and 17 in BPD through anatomopathological and immunohistochemical study of the lungs of premature neonates with BPD. **Method:** Thirty two cases of neonatal necropsies from the Pathology Department of the Clinics Hospital of the Universidade Federal do Paraná performed between 1991 and 2005 were selected. The sample included neonates of less than 34 weeks of gestational age who underwent oxygen therapy and had pulmonary samples fixed in formalin and preserved in paraffin. Pulmonary specimens were later classified into 3 groups according to histopathological and morphometric changes (classic BPD, new BPD, and without BPD) and subjected to immunohistochemical analysis. The antibodies selected for the study were anti-IL-6, IL-8, IL-10 and IL-17 monoclonal antibodies. **Results:** IL-6, IL-8 and IL-10 showed no significant differences in tissue immunoexpression between the groups. IL-17 had greater tissue immunoreactivity in the group without BPD compared to the classic BPD group (1686 vs 866 μm^2, p = 0.029). **Conclusion** This study with lungs tissue of premature neonates with BPD showed that ILs-6, 8, 10 and 17 didn´t play significant role in the development of BPD.

LISTA DE ABREVIATURAS E SIGLAS

%- porcentagem

g- grama

μm^2- micrômetro quadrado

CCL5- ligante de citocina 5

DBP- diaplasia broncopulmonar

ICAM-1- molécula de adesão intercelular-1

IL-interleucina

ILs- interleucinas

IL-6- interleucina 6

IL-8- interleucina 8

IL-10- interleucina 10

IL-17- interleucina 17

IL-17A- interleucina 17A

LPA- lesão pulmonar aguda

MCP-1- proteína quimioatraente de monócito

RANTES- regula ativação célula T normal espressa e secretada

SDRA- síndrome do distress respiratório agudo

Th17- linfócito T helper 17

TNF-α- fator de necrose tumoral α

TNF-β- fator de necrose tumoral β

SUMÁRIO

1. INTRODUÇÃO E REVISÃO DE LITERATURA

A displasia broncopulmonar (DBP) é uma doença pulmonar crônica que acomete neonatos prematuros, quando expostos ao tratamento com oxigênio e, ou ventilação mecânica por tempo prolongado. Estes insultos levam a um desenvolvimento pulmonar alterado que pode culminar com insuficiência respiratória persistente, prolongando-se, muitas vezes, pelos primeiros anos de vida.

É definido clinicamente com DBP os neonatos, que recebem oxigênio complementar por no mínimo 28 dias (JOBE, 2001; GOMEZ, 2018) ou que estão em uso de oxigênio crônico na 36° semana de idade corrigida (idade pós-concepção) (SHENNAN, 1988). Podendo apresentar desde mínima alteração pulmonar, até insuficiência respiratória grave (TANNURI, 2004).

O nascimento prematuro é aquele que ocorre antes da 37° semana gestacional. O desenvolvimento pulmonar é dividido em 5 estágios: embrionário (3-7 semanas), pseudoglandular (5-17 semanas), canalicular (16-26 semanas), sacular (24-38 semanas) e alveolar (a partir de 32 semanas até 18 meses). No final do estágio canalicular há o desenvolvimento do alvéolo primitivo, do capilar alveolar e a diferenciação dos pneumócitos tipo I e tipo II. No início do estágio sacular começa a produção de surfactante, a vascularização pulmonar e o alargamento da via aérea terminal. O nascimento prematuro ocorre geralmente durante o estágio canalicular ou sacular (KALLAPUR, 2006; BHANDARI, 2016). Sendo então os recém-natos mais acometidos os com idade gestacional inferior a 30 semanas e menores que 1500g, devido à imaturidade pulmonar (JOBE, 2016).

No mundo, tem-se 15 milhões de nascimentos prematuros por ano, sendo a prematuridade uma das maiores causas de incapacidade no ser humano nos dias de hoje. Cerca de 25% dos prematuros que nascem com peso menor que 1500g evoluem com DBP (O´REILLY, 2013; JENSEN, 2014; PURISCH, 2017).

Nos últimos anos, nos Estados Unidos e Canadá, o aumento da prematuridade foi de 35% devido ao crescimento do número de reprodução assistida, das gestações em idades mais avançadas e da melhoria na assistência perinatal. (DIMES, 2009) Em 2015, segundo o National Center for Health Statistics, o índice de nascimentos prematuros nos Estados Unidos foi de 9,6%. Sendo que a DBP se destaca como a mais onerosa entre as morbidades neonatais (JONHSON, 2013; PURISCH, 2017).

No Brasil, a prevalência de nascimentos prematuros entre os anos de 2011 e 2012 foi de 11,5% (LEAL, 2016). Segundo estudo realizado pela Rede Brasileira de Pesquisas Neonatais no ano de 2015, 14% dos recém-natos com peso de nascimento menor que 1500g, usavam oxigênio no 36° semana de idade corrigida.

A primeira descrição da DBP foi feita por NORTHWAY (1967), como uma doença pulmonar crônica, encontrada em prematuros com síndrome do desconforto respiratório, submetidos à ventilação mecânica prolongada. Os prematuros em questão foram expostos a pressões e frações inspiratórias de oxigênio (FiO2) elevadas, necessitando de oxigênio por meses ou anos. Após, no ano de 1969 PURSEY descreveu casos de DBP em crianças com doenças pulmonares de etiologias variadas, e não apenas na síndrome do desconforto respiratório. Em 1988, SHENNAN introduziu a nomenclatura “doença pulmonar

crônica do prematuro", para aqueles que necessitassem de oxigênio complementar após 36° semanas de idade corrigida, com sintomas e sinais respiratórios, além de alterações radiológicas características. No ano de 2001, o Instituto Nacional de Saúde da Criança e Desenvolvimento Humano fixou a terminologia displasia broncopulmonar- DBP em detrimento à "doença pulmonar crônica**".** Definiu com DBP aquele neonato que necessita de no mínimo 28 dias de oxigênio complementar.

Portanto o diagnóstico clínico fica dependente da espera de um dia específico para ser definido, o que não ocorre no diagnóstico anatomopatológico, o qual reflete e define o achado do momento da coleta. Porém, a punção transtorácica é contraindicada em pacientes submetidos a ventilação mecânica, e está relacionada a complicações como pneumotórax e hemotórax, além de ter uma sensibilidade de 60% e não oferecer um material adequado para avaliação histológica, sendo então seu uso limitado na pediatria. BOUSSO (1998) estudou 26 casos de biópsia a "céu aberto" em crianças e concluiu que o procedimento tem um potencial elevado de diagnóstico. No entanto, a biópsia pulmonar em lactentes com peso menor que 6kg tem risco muito aumentado, tendo suas complicações multiplicadas em neonatos com baixo peso. E o lavado broncoalveolar que seria menos invasivo, não esclarece o diagnóstico eficazmente. Por conseguinte, a análise anatomopatológica é praticamente observada apenas em material de necropsia.

A apresentação anatomopatológica da DBP tem duas formas: a clássica e a nova. A clássica é caracterizada por: inflamação pulmonar, dano secundário de via aérea por sobrecarga hídrica alveolar, fibrose do parênquima

pulmonar e hipertrofia do músculo liso. Essa forma é pouco comum nos dias de hoje, devido ao uso de novas técnicas terapêuticas como ventilação mecânica menos agressiva, uso de corticoide antenatal, administração de surfactante, manejo adequado do neonato com persistência do canal arterial, uso de cafeína e óxido nítrico e mudanças no atendimento na sala de parto. Esses cuidados no manejo do prematuro ofuscaram a incidência da DBP clássica, dando lugar a DBP nova (O´RYELLY, 2013; CERNY, 2008; BARALDI, 2007). Porém, a DBP clássica ainda pode ser encontrada em pacientes que necessitam de ventilação com altos níveis de oxigênio e pressão (MONTE, 2005). A DPB nova é caracterizada pela diminuição da incidência da fibroproliferação, menor grau de lesão de vias aéreas, inflamação pulmonar mais difusa, redução do crescimento alveolar distal com menor número de alvéolos (hipoalveologênese) e inibição do desenvolvimento da microvasculatura pulmonar (MONTE, 2005; CERNY, 2008; BHANDARY, 2006). Portanto, as duas formas da DBP apresentam sinais de inflamação, sendo que na DBP clássica essa característica é mais pronunciada.

O papel do oxigênio na fisiopatologia das doenças pulmonares é amplamente estudado. O oxigênio é responsável pelas reações de oxidação, porém nem sempre a reação de oxidação fosforilativa resulta na formação de água e sim forma produtos intermediários como o ânion superóxido, o peróxido de hidrogênio e o radical hidroxila. Esses produtos são agentes oxidantes fortes, e altamente reativos, necessitando da existência de enzimas que os neutralize. Elas nem sempre estão presentes (devido a prematuridade) e se estão, às vezes não são suficientes para conter as reações oxidativas, as quais destroem vários componentes celulares como as proteínas, membranas

lipídicas e ácidos nucléicos. Resultando na ruptura das membranas celulares, liberação de enzimas degradadoras e necrose. A ação das substâncias oxidativas sobre o pulmão é o aumento da produção de mediadores inflamatórios, os quais danificam o pulmão por possuir atividade vasoativa e broncoativa, aumentam a permeabilidade vascular pulmonar e atraem leucócitos e outras células inflamatórias para o pulmão (TANNURI, 2004).

Em relação à ventilação mecânica e as doenças pulmonares, especificamente na DBP, a ventilação causa lesão celular devido aos mecanismos de barotrauma, biotrauma, atelectrauma e volutrauma. Logo após o nascimento, quando ocorre a administração da ventilação mecânica, há um influxo de neutrófilos e macrófagos no interstício pulmonar via capilar alveolar. Estas células inflamatórias produzem as citocinas em resposta ao dano tecidual. No entanto, há uma subsequente exacerbação da lesão celular, na unidade alvéolo-capilar, induzindo a apoptose de células endoteliais e alveolares que participam deste processo inflamatório, e na prossecução, ocorre uma tentativa imperfeita de regeneração (RYAN, 2008; SPEER, 2006; KALLAPUR, 2006; CORDERO, 2001; SPEER, 2003).

Quando essa exposição aos mediadores inflamatórios, tanto decorrentes do uso do oxigênio quanto da ventilação mecânica, ocorre no período crítico da alveologênese, o pulmão não recupera totalmente seu potencial de crescimento, gerando hipoalveologênese e hipoplasia da vasculatura pulmonar (MASSARO, 2004).

A análise histopatológica de pulmões de prematuros com diagnóstico de DBP mostra então, alterações do desenvolvimento do parênquima, das vias aéreas de condução e da vasculatura pulmonar. E a avaliação pulmonar destes

mesmos indivíduos adultos revela que o déficit de alveolarização persiste, proporcionando uma diminuição da função pulmonar devido a redução das superfícies de trocas gasosas. Essa diminuição da capacidade funcional pulmonar acarreta em uma maior suscetibilidade a doenças respiratórias infecciosas e a hipertensão pulmonar, tanto na infância como na vida adulta (O´REILLY, 2013).

Na atualidade, a DBP é conhecida como a mais importante causa de morbidade e mortalidade no período neonatal e uma das principais razões de doença respiratória crônica na infância. Além da suscetibilidade as doenças pulmonares, alteração do crescimento pôndero-estatural e do desenvolvimento neuropsicomotor, as crianças com DBP tendem a ser submetidas a várias internações ao longo da vida, justificando assim a relevância clínica e de saúde pública desta doença (JOBE, 2001; MONTE, 2005; KINSELLA, 2006).

A DBP tem etiologia multifatorial, além da exposição à ventilação mecânica e ao oxigênio, que isoladamente já são capazes de impedir a septação pulmonar (COALSON, 1995), tem-se também a predisposição genética, restrição de crescimento intrauterino, déficit de surfactante pulmonar, presença de infecção pré ou pós-natal e a persistência do canal arterial patente. A maioria deles desencadeia uma resposta inflamatória, lesão, alteração e inibição da maturação e desenvolvimento das superfícies de trocas alveolares, levando as manifestações clínicas da DBP (THOMPSON, 2008; RYAN, 2008; BHANDARI, 2007; JOBE, 1998; SPEER, 2004; LISTA, 2006; AHLFELD, 2012; COUROCLI, 2000; BEM-ARI, 2000; JENSEN, 2014).

O processo inflamatório persistente pode ser o fator que mais contribui para o desenvolvimento de doenças crônicas pulmonares (SU, 2005).

Evidências acumuladas sugerem que a inflamação tem um grande papel na patogênese da DBP (DEAKINS, 2009; RYAN, 2008; PIERCE, 1995; BANCALARI, 2001; ROCHA, 2012). A resposta inflamatória num pulmão ainda em formação causa alteração do crescimento vascular, alveolar e brônquico (LAUGHON, 2009; KUNZMANN, 2013; CHOI, 2008). A incapacidade dos prematuros de regular a inflamação é um fator que contribui para o desenvolvimento da DBP (SCHULTZ, 2004). Sendo assim, a DBP é o produto final da resposta inflamatória e da tentativa de reparação do tecido pulmonar frente à injúria, tendo como resposta a hipoalveolização e alteração da vascularização pulmonar (BHANDARI, 2006).

Os neonatos com DBP têm uma maior concentração de interleucinas (ILs) no aspirado traqueal, sangue do cordão umbilical, líquido amniótico e plasma. E as ILs em maiores quantidades são a interleucina 6 e 8 (IL-6 e IL-8) (THOMPSON, 2008; AN, 2004). Estudos feitos com aspirado traqueal coletado nos primeiros dias de vida encontraram altas concentrações de marcadores inflamatórios nos recém-natos que desenvolveram a DBP, sendo eles: o neutrófilo, o macrófago, o leucotrieno, o fator ativador de plaqueta, as IL-6, IL-8 e o fator de necrose tumoral (TNF) (GRONECK, 1994; MIRRO, 1990; CHESS, 2006; JONSSON, 2000; JONSSON, 1997).

Pesquisas demonstram que a DBP resulta da presença de citocinas pró-inflamatórias e macrófagos, alterando o mecanismo do desenvolvimento alveolar (RYAN, 2008; RAMANATHAN, 2001). Acredita-se que há um desequilíbrio entre fatores “pró” e “anti-inflamatórios”, esse processo é caracterizado por células inflamatórias, citocinas e um arsenal de mediadores humorais nas vias aéreas e no tecido pulmonar do prematuro. Em um estudo

feito com camundongos observou-se uma expressão aumentada de citocinas, TNF-β, TNF-α, IL-6 e IL-11 no espaço aéreo pulmonar interferindo na formação do alvéolo, sugerindo que a presença de fatores pró-inflamatórios no pulmão do prematuro, contribui para alteração no processo de septação pulmonar. (JOBE,1999) Outro estudo com aspirado traqueal indicou que as interleucinas IL-1β, IL-6, IL-8 e IL-16 (interleucinas pró-inflamatórias) estão elevadas precocemente nos prematuros que irão desenvolver DPB e nos que possuem a doença (THOMPSON, 2008).

Em 2009, o Instituto Nacional de Saúde da Criança e Desenvolvimento Humano realizou um trabalho analisando a associação de citocinas com DBP e/ou morte em prematuros extremos. Encontraram que as citocinas (IL-1β, IL-6, IL-8, IL-10, TFN-α) em altas concentrações plasmáticas estavam associadas à DBP e morte. Já as citocinas (IL-17, RANTES/CCL5 e TNF-β) em altas concentrações estavam presentes nos neonatos que sobreviveram sem a doença. É possível que essas citocinas possam servir como marcadores de lesão pulmonar grave ou de estarem envolvidas na patogênese da DBP. Altos níveis séricos de IL-6 encontrados entre o 14º e 21º dia de vida associaram-se a um maior número de DBP acompanhada de óbito, sendo que essa citocina é a única com esta característica neste período. Todavia as diversidades e interferências nas concentrações das citocinas ao longo do período neonatal mostram que uma aferição única, num ponto do tempo, não consegue definir prognóstico para DBP (AMBALAVANAN, 2009; BOHRER, 2010).

A IL-6 é uma citocina com importante papel na resposta inflamatória aguda, infecção, hematopoiese, regulação da absorção óssea, células de crescimento, diferenciação, sobrevivência, apoptose e proliferação (HEINRICH,

2003; XING, 1998; DAME, 2000). A IL-6 é produzida por monócitos, fibroblastos e células endoteliais e está presente em doenças agudas pulmonares (KISHIMOTO, 2006).

DAME e JUUL (2000) provaram que o receptor da IL-6 está amplamente distribuído no tecido fetal, incluindo as células do epitélio brônquico. Altos níveis de RNA mensageiro da IL-6 foram encontrados no pulmão fetal na fase pseudoglandular, diminuindo nos subsequentes estágios (SILVA, 2006).

VON BISMARCK (2008) concluiu que a IL-6 tem um papel importante na inflamação pulmonar de recém-natos prematuros. Analisou o nível de concentração de IL-6 no aspirado traqueal dos prematuros submetidos à ventilação mecânica, e ela estava aumentada na primeira semana de vida. GOMEZ (1998) observou níveis aumentados de IL-6 no sangue de cordão, identificando uma resposta inflamatória sistêmica fetal, esses indivíduos possuíam maior risco de desenvolver morbidades como a síndrome do distress respiratório, sepse, pneumonia, hemorragia intraventricular, enterocolite necrotizante e DBP. HSIAO (2017) reafirmou esta análise observando níveis aumentados e persistentes de IL-6 no aspirado traqueal de prematuros extremos, submetidos à ventilação mecânica, até no 28º dia de vida. Sendo assim, altos níveis de IL-6 no aspirado traqueal são considerados como fator de risco independente para o desenvolvimento da DBP.

Porém existem linhas de conflito no estudo da IL-6, um estudo demonstrou efeito de proteção celular da IL-6 frente à exposição ao oxigênio em tecido pulmonar adulto de camundongos, esse resultado pode ter sido encontrado por se tratar de um pulmão maduro. (WARD, 2000) Outro estudo já em fetos com corioamnionite, expôs que a elevação da IL-6 promove a

maturação pulmonar por meio da promoção da síntese da proteína surfactante A. A IL-6 fetal é uma citocina reguladora das proteínas do surfactante pulmonar, sendo muito importante na maturação pulmonar. Também regula a angiogênese e morfogênese do pulmão, diminuindo a incidência da síndrome do distress respiratório do prematuro (SHIMOYA, 2000; McCLINTOCK, 2005; PARERA, 2005).

Portanto todas estas observações a respeito da IL-6 reafirmam a etiologia multifatorial da DBP e necessitam de mais estudos sobre a ação dela em tecidos imaturos expostos ao oxigênio.

A IL-8 é produzida por leucócitos, macrófagos alveolares, fibroblastos, células endoteliais, células epiteliais quando o organismo é exposto a patógenos e agentes estressores como o oxigênio (MUKAIDA, 2003). É um potente agente quimiotáxico capaz de recrutar neutrófilos, e a presença de neutrófilos no aspirado traqueal está associada a doenças crônicas pulmonares (SU, 2005). O aumento da expressão da IL-8 no aspirado broncoalveolar pode estar associado a um desenvolvimento e maturação pulmonar anormal nos neonatos com DBP (LIU, 2010; CHAKRABORTY, 2014) e um maior tempo de permanência em ventilação mecânica (DE DOOY, 2007). ROCHA (2012) concluiu que a IL-8 elevada no sangue de cordão estava associada com morte e DBP moderada e severa. Em adultos, observou-se um aumento da IL-8 nos pacientes que eram ventilados com volume corrente alto, ou seja, aqueles sujeitos ao volutrauma (PARSON, 2005).

A IL-10 é uma citocina anti-inflamatória potente (SPEER, 2003; HAWWA, 2011). É produzida por macrófagos e linfócitos T-helper tipo II que fazem a regulação da produção de mediadores inflamatórios, através da

estimulação de células epiteliais do sistema imune (BAIER, 2006). São responsáveis pela produção, diferenciação e proliferação de células B e macrófagos (SPEER, 2003).

Níveis reduzidos de IL-10 na placenta e no aspirado traqueal são encontrados em neonatos que desenvolveram DBP (MCGOWAN, 2009; GARINGO, 2007). HAWWA em 2011 demonstrou que o estiramento mecânico das estruturas pulmonares causado pela ventilação mecânica provocou a liberação de IL-1β, IL-6, MCP-1, RANTES/CCL5 e TNF-α e a administração de IL-10 antes do estiramento bloqueou a liberação dos mesmos. Concluindo que a IL-10 protegeu o pulmão de prematuros submetidos à ventilação mecânica. Ainda em 2011, LEE evidenciou o aumento da necrose celular e da produção de IL-8 frente à hiperóxia, e a diminuição da produção da IL-10 e da proliferação celular diante desde insulto. A incubação celular prévia com IL-10 recombinante foi um fator de proteção contra a injúria causada pelo oxigênio. A IL-10 melhora a oxigenação do tecido pulmonar e inibe o estress oxidativo, diminuindo a lesão induzida pela ventilação mecânica e a inflamação (CHEN, 2018; MOSSER, 2008). DAVIDSON (2013) também estudou o uso da IL-10 como inibidora da cascata inflamatória na DBP, essa interleucina mostrou-se mais potente para bloquear a IL-8, do que os glicocorticóides utilizados na terapêutica atual da doença.

Alguns prematuros não são hábeis para ativar citocinas anti-inflamatórias, como a IL-10, assim sendo estão mais predispostos a uma resposta inflamatória mais acentuada (DE DOOY, 2001).

Já estudos com prematuros extremos mostraram que a expressão da IL-10 no aspirado pulmonar é aumentada logo após o nascimento e relaciona-se a

incidência de DBP e sua expressão aumentada juntamente com a presença de corioamnionite também eleva a incidência da doença (HIKINO, 2012). A dosagem sérica alta da IL-10 foi relacionada à DBP associada à morte (AMBALAVANAN, 2009). Pesquisa feita com a dosagem sérica de IL-10 e IL-8 no cordão umbilical no primeiro dia de vida mostrou níveis elevados destas interleucinas, sendo associado a um maior risco de desenvolvimento de DBP (PAANANEN 2009). Estudo genético feito com IL-6 e IL-10 mostrou não haver relação destas interleucinas com a DBP (HUUSKO, 2014).

A IL-17 é uma citocina pró-inflamatória, pertencente à família de seis ILs (IL17A-F), ela auxilia na defesa do sistema imune contra patógenos extracelulares e é produzida pelo linfócito T (Th17) (RAJITA, 2011; LAWRENCE, 2018) e por células imunes inatas. Essas células estão estrategicamente localizadas em tecidos de barreira que protegem o corpo humano do ambiente externo e fornecem proteções vitais ao hospedeiro contra microorganismos (principalmente bactérias e fungos) (CARON, 2014; HUANG, 2004). Em 4 a 8 horas após a exposição aos microoganismos, as células imunes inatas são hábeis a aumentar a produção de IL-17A, atraindo neutrófilos para locais inflamados a fim de acelerar a morte e eliminação de microrganismos agressores (CUA, 2010). A IL-17 tem sido implicada no desenvolvimento ou patogênese de várias doenças autoimunes e inflamatórias (SAKAGUCHI, 2016).

Os neonatos tem uma produção basal de IL-17A reduzida em comparação aos adultos, o que pode diminuir a resposta imune neonatal e contribuir para sua maior suscetibilidade à infecção (SCHELONKA, 2010; SOOD, 2012; LAWRENCE, 2018) pelo Streptococcus do grupo B, Escherichia

coli (CARON, 2014), Klebsiella pneumoniae (HAPPEL, 2003) e Candida albicans (HUANG, 2004). Os níveis de IL-17A são semelhantes entre recém-nascidos pré-termo e a termo, sua produção não depende da maturação imunológica ou do avanço da idade gestacional (SCHELONKA, 2010). A produção de IL17A no timo embrionário inicia-se por volta da 24°-28° semanas de gestação. (LAWRENCE, 2018).

Processos inflamatórios maternos, como a corioamnionite podem modificar a imunidade pós-natal e aumentar a resposta inflamatória sistêmica nos prematuros. (GLEDITSH, 2014). Os linfócitos Th17 produtores de IL-17A estão aumentados no sangue do cordão em prematuros cujas mães tinham corioamnionite (RITO 2017). A IL-17 é importante para a vigilância imunológica e proteção do neonato, há evidências de que a IL-17 desempenha um papel proeminente na patologia neonatal. (LAWRENCE, 2018). MIKACENIC, em 2016, demonstrou que a IL-17 sérica e alveolar estava elevada em individuos com síndrome do distress respiratório agudo (SDRA), mas o papel da resposta imune Th-17 na lesão pulmonar aguda (LPA) / SDRA ainda não foi definido. O anticorpo anti-IL17 tem demostrado alguma eficácia na proteção de pulmões diante de processos inflamatórios agudos. (RIGHETTI, 2018; LI, 2017). Já um estudo sobre sepse e IL-17 não relacionou a presença da mesma com o processo inflamatório (SOOD, 2012). E outro feito por AMBALAVANAN em 2009, constatou que a baixa dosagem sérica de IL-17 estava associada à DBP/morte, sugerindo que a redução sérica de IL-17 possa estar associada à possibilidade de inibição da angiogênese e do desenvolvimento alveolar.

Ainda não se sabe o real papel desta IL na DBP. Sabe-se que IL-17A contribui para a manutenção de uma borda epitelial respiratória saudável,

promovendo a produção de moléculas de adesão e junção celular por meio da indução da ICAM-1 pelas células epiteliais das vias aéreas (KAWAGUCHI, 2001; KINUGASA, 2000; TSAI, 2013). Portanto, a regulação rigorosa da IL-17A parece vital para a manutenção adequada da borda do epitélio respiratório, enquanto a produção alterada pode prejudicar o desenvolvimento do tecido pulmonar, resultando em patologias pulmonares prejudiciais nos neonatos (LAWRENCE, 2018).

A regulação da IL17 é fundamental para a homeostase do indivíduo. O seu aumento é realmente deletério (processos inflamatórios permanentes) ou sua ausência poderia ser fatal (dificuldade em combater microoganismos extracelulares). A prematuridade ainda é um desafio para a ciência, o papel das IL nas doenças neonatais necessita de mais pesquisas, em busca de biomarcadores e instrumentos terapêuticos. Existe um interesse crescente no estudo de diferentes marcadores biológicos para verificar a suscetibilidade e, ou gravidade da DBP (BHANDARI, 2013). Sugere-se que os eventos iniciais da DBP ocorram precocemente, logo após o nascimento, e o seu diagnóstico está limitado a aguardar o 28° dia de vida ou constatar as morbidades e até mortalidade, para então identificar a presença da patologia (DEAKINS, 2009; SPEER, 2003).

O local de coleta da interleucina constitui um importante fator a ser analisado. O perfil das citocinas no sangue do cordão, líquido amniótico e aspirado traqueal pode não refletir o que realmente se passa nos pulmões, portanto a questão de estudar concentrações de biomarcadores pulmonares, e não apenas os séricos, seria o ideal, pois os pulmonares representariam melhor a realidade da doença (SPEER, 2003).

O estudo da resposta inflamatória no material de necropsia tem a vantagem de ser um material controlado e com análise imparcial dos dados, porém a desvantagem consiste no preparo do material, que é fixado em formol e emblocado em parafina, tornando mais difícil a pesquisa de moléculas secretadas, pequenas e frágeis como as interleucinas (MCFARLANE, 1987; FORGHANI, 1987, SIMON, 2004).

A existência de um marcador poderia identificar e alertar a equipe médica, auxiliando nos cuidados para que esses recém-natos não desenvolvam, ou minimizem as manifestações desta doença. A identificação das citocinas que contribuem para a DBP, ou para a associação DBP e morte, poderiam levar a pesquisas dirigidas contra essas citocinas e seus receptores (RYAN, 2008; SMOLEN, 2006; YAMAGATA, 2006).

A atuação das interleucinas na patogênese da DBP não está esclarecida, e pesquisas com tecido pulmonar humano são escassas. Com o aumento da sobrevivência de prematuros extremos, o número de adolescentes e adultos com sequelas da DBP aumentou (HULSMANN, 1997). Devido ao impacto em longo prazo, tanto na vida do indivíduo como da sociedade, pesquisas para elucidar melhor a fisiopatogenia, fatores genéticos e biomarcadores da DBP são importantes para que possam ser desenvolvidas medidas profiláticas e terapêuticas (TAPIA, 2006).

Este estudo se baseia no fato do desconhecimento do papel das IL-6, IL-8, IL-10 e IL-17 na DBP, da ausência de biomarcadores para esta patologia e na escassez de trabalhos com tecido pulmonar humano.

2. OBJETIVO

2.1 OBJETIVO GERAL

Avaliar o envolvimento das IL-6, IL-8, IL-10 e IL-17 na DBP, através do estudo anatomopatológico de pulmões de neomortos prematuros com DPB clássica e nova.

2.2 OBJETIVO ESPECÍFICO

Correlacionar o padrão de expressão tecidual das IL-6, IL-8, IL-10 e IL-17 de cada grupo com os:

- dados clínicos pré-natais (amniorexe prematura, corioamnionite, diabetes gestacional, doença hipertensiva específica da gestação);

- dados clínicos pós-natais (tempo de vida, tempo de uso de oxigênio, Apgar de primeiro e quinto minuto, idade gestacional, Parkin, peso de nascimento, enterocolite necrotizante, pneumonia, persistência do canal arterial, sepse, asfixia perinatal, uso de antibiótico, hemorragia pulmonar, hipertensão pulmonar).

3. NOTA EXPLICATIVA

Esta tese faz parte da linha de pesquisa: ASPECTOS CELULARES E MOLECULARES DA DOENÇA HUMANA. A doença estudada foi a Displasia Broncopulmonar (DBP). E as interleucinas 6, 8, 10 e 17 foram o alvo da investigação para tentar ajudar na elucidação da fisiopatogenia desta doença, bem como, no futuro, contribuir com possíveis novos métodos diagnósticos e/ou terapêuticos. Três artigos foram desenvolvidos, sendo intitulados:

1- **IMMUNOHISTOCHEMICAL ANALYSIS OF APOPTOSIS AND CELL PROLIFERATION IN LUNGS OF PREMATURE INFANTS WITH CHRONIC LUNG DISEASE (BRONCHOPULMONARY DYSPLASIA)**

2- **QUANTITATIVE ANALYSIS OF INFLAMMATORY AND ADHESION MOLECULES IN LUNGS OF NEONATES WITH CHRONIC LUNG DISEASE (BRONCHOPULMONARY DYSPLASIA) RECEIVING MECHANICAL VENTILATION**

3- **ANALYSIS OF INTERLEUKINS 6, 8, 10 AND 17 IN THE LUNGS OF PREMATURE NEONATES WITH BRONCHOPULMONARY DYSPLASIA**

O primeiro artigo fez parte da dissertação do meu mestrado, publicado no Jornal Brasileiro de Patologia e Medicina Laboratorial. v. 52, n. 6, p. 407-415, 2016.

O segundo artigo foi realizado com outros membros do nosso grupo, sendo produto de dissertação de mestrado e tese de doutorado dos mesmos. O artigo foi publicado no Jornal Brasileiro de Patologia e Medicina Laboratorial. v. 52, n 4, 2016.

O terceiro artigo “Análise das interleucinas 6, 8, 10 e 17 em pulmões de neonatos prematuros com displasia broncopulmonar”, é o resultado da pesquisa sobre broncodisplasia e interleucinas, exposto nesta tese. O artigo já foi submetido à uma revista internacional de pneumologia pediátrica, atualmente em análise, já na primeira correção**.**

4. ARTIGOS

4.1 ARTIGO 1

IMMUNOHISTOCHEMICAL ANALYSIS OF APOPTOSIS AND CELL PROLIFERATION IN LUNGS OF PREMATURE INFANTS WITH CHRONIC LUNG DISEASE (BRONCHOPULMONARY DYSPLASIA).

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Immunohistochemical analysis of apoptosis and cell proliferation in lungs of premature infants with chronic lung disease (bronchopulmonary dysplasia)

Análise imuno-histoquímica da apoptose e proliferação celular em pulmões de prematuros com doença pulmonar crônica (displasia broncopulmonar)

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ABSTRACT

Introduction: The pathophisiology of chronic lung disease (CLD), clinically known as bronchopulmonary dysplasia is not clear. It is believed that protective mechanisms, such as the release of inflammatory mediators and the activation of apoptotic and/or proliferative processes are activated in the lung tissue of premature infants in an attempt to repair tissue injury caused by exposure to oxygen and mechanical ventilation. **Objective:** Assess the presence of apoptosis and cell proliferation in the lungs of premature infants with CLD, exposed to oxygen and/or mechanical ventilation, by analyzing the proteins expression: proliferating cell nuclear antigen (PCNA), phosphatase and tensin homolog (PTEN), B-cell lymphoma 2 (Bcl-2), tumor necrosis factor receptor family member (Fas), fas-associated protein with death domain (FADD), tumor necrosis factor receptor type 1-associated death domain protein (TRADD), Caspase 3 and Caspase 8. **Material and methods:** We analyzed 32 infants autopsies at gestational age of less than 34 weeks exposed to oxygen therapy. The study was divided into three groups: "classic" CLD, "new" CLD and "without" CLD. Immunohistochemical analysis was performed. **Results and discussion:** A higher proliferation rate was observed in infants with CLD suggesting that longer exposure to mechanical ventilation may stimulates cell proliferation. The PTEN and Caspase 8 expressions were higher in the "new" CLD group, compared to the "without" CLD group, indicating that the "new" CLD form is more susceptible to apoptosis. **Conclusion:** Apoptosis and cell proliferation are involved in the pathophisiology of CLD. The "new" CLD form is more susceptible to apoptosis, while cell proliferation is more evident in the groups with CLD.

Key words: apoptosis; pharmacological biomarkers; cell proliferation; bronchopulmonary dysplasia; neonatology.

INTRODUCTION

The pathophysiology of bronchopulmonary dysplasia (BPD) can be the key for prevention of this disease, research with cell cycle proteins appear to be very promising for elucidating this pathology.

Apoptosis and cell proliferation are controlled physiological tissue repair processes regulated by mediators and proteins. Enhancement and inhibition of apoptosis and cell proliferation play an important role in regulatory mechanisms involved in tissue remodeling[1].

In an attempt to repair tissue injury in the lungs of premature infants exposed to oxygen and mechanical ventilation, protective mechanisms, such as the release of inflammatory mediators and the activation of apoptotic and/or proliferative processes, are triggered[2-4]. These mechanisms seem to be part of the pathophysiology of BPD.

BPD is a chronic lung disease (CLD) affecting mainly low-weight newborns (particularly those weighing less than 1500 g) that frequently receive mechanical ventilation and/or oxygen therapy. Neonates are clinically defined as having BPD/CLD if they still need oxygen supplementation at 28 days of life (or respiratory support at 36 weeks corrected age)[5]. However, the pathological

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alterations of BPD/CLD can be observed at an earlier age, before 28 days of life, and they are based on the presence of the classic characteristics of exacerbated inflammatory process, over-distended acini, atelectasis, fibrosis, squamous metaplasia and airway and vascular smooth-muscle hypertrophy in the "classic" form. With the use of antenatal steroids, exogenous surfactant and less aggressive mechanical ventilation in recent decades, another form of BPD, known as "new" BPD, have emerged to replace the "classic" form. The fundamental histological characteristic of "new" BPD/CLD is reduced alveolarization. The mechanisms involved in this abnormal alveolar development are not yet well understood[6-8].

As cell proliferation and apoptosis can adversely affect lung-tissue homeostasis, the efforts to elucidate the pathophysiology of "new" BPD/CLD have involved research on the cell-cycle proteins, since this type of BPD/CLD appears to be the result of interrupted distal lung growth[9, 10].

Studies in animal models and cell cultures show that apoptosis increases in the lungs of premature infants exposed to oxygen and mechanical ventilation[11-15]. Although few studies have been carried out on humans[10, 16].

The aim of this study is to investigate the expression of the proteins involved in the cell-cycle [proliferating cell nuclear antigen (PCNA), phosphatase and tensin homolog (PTEN), B cell lymphoma 2 (Bcl-2), death receptor (Fas), Fas-associated protein with death domain (FADD), tumor necrosis factor receptor type 1-associated death domain protein (TRADD), cysteine-aspartic acid protease 3 (Caspase 3) and cysteine-aspartic acid protease 8 (Caspase 8)] in lung autopsy samples from premature infants that required assisted ventilation, with pathological evidence of "classic" or "new" CLD, and compare them to the expression of the same proteins in newborns without pathological evidence of CLD.

MATERIAL AND METHODS

All 466 autopsy reports on infants who died in the neonatal period were reviewed by the Anatomic Pathology Service, Hospital de Clínicas (HC), Universidade Federal do Paraná (UFPR), between 1991 and 2007 (post exogenous surfactant era). The ethics review board of this hospital reviewed and approved the study (Register number 1099.138/2005-08; approved on 30 August 2005). Only premature infants with a gestational age between 25-34 weeks who were submitted oxygen therapy were included in the study. Premature infants with congenital abnormalities, chronic intrauterine diseases or meconium aspiration syndrome, as well as those whose medical records or samples were inadequate, were excluded. Thirty-two cases were included in this study.

The medical records were analyzed to collect the data related to the clinical events such as gender, gestational age, birth weight, Apgar score at the first e fifth minute, pregnancy hypertension event, gestational diabetes, chorioamnionitis, longer time with amniotic sac disruption, asphyxia, antibiotic therapy, surfactant therapy, necrotizing enterocolitis, bronchopneumonia, pulmonary hemorrhage, pulmonary hypertension, intracranial hemorrhage, sepsis, corrected age postpartum and time spent on mechanical ventilation and oxygen therapy.

Morphometric analysis

The formalin-fixed paraffin-embedded lung tissue samples were reexamined and classified into one of the three groups according to the histopathological and morphometric changes (without considering the clinical data): A) the histopathological findings of "classic" CLD ($n = 11$); B) the histopathological and morphometric findings of "new" CLD ($n = 5$); and C) "without" histopathological and morphometric criteria for CLD ($n = 16$). As the alterations in alveolar formation could be very subtle, the samples in groups B and C were analyzed only to confirm the existence of the morphometric criteria of "new" CLD in the group B[17-20], data are shown in **Table 1**.

Olympus BX 50 microscope (Olympus Optical Co. LTDA, Japan) linked to a Dino eye camera and a computer were used to obtain 10 medium power field photomicrographs (200×) per case (the total area of medium power field is 475.439 square micrometers). These photomicrographs (200×) were subjected to the morphometric analysis, using Image Pro Plus software. All alveoli in each medium power field were counted and their perimeters were measured (micrometers). Mean values (number of alveoli and perimeter) for each patient were used for statistical analysis.

Immunohistochemistry staining

Tissue microarrays were collected from lung samples from all the cases and analyzed immunohistochemically (four samples for each case with 3 mm diameter each), since this is the most suitable technique to analyze protein expression in this type of material[17, 21].

The proteins used in this study were: PCNA is associated with cell proliferation, Bcl-2 is associated with resistance to

TABLE 1 – Pathological data for the groups

	Classic CLD (n = 11)	New CLD (n = 5)	Without CLD (n = 16)	p-value
Pathological characteristics	Hyaline membrane, interstitial fibrosis and over-distended acini	Slight septal edema, low-grade inflammation	Slight septal edema, low-grade inflammation	
Morphometry (number of alveoli)*	-	40.94/15.13	55.8/15.1	0.050[a]
Morphometry (alveolar perimeter)*	-	481.52/182.42	560.4/168.8	0.548[a]

*CLD: chronic lung disease; *: mean µm²/standard deviation; [a]: nonparametric Mann-Whitney test,* $p < 0.05$.

apoptosis, PTEN is associated with susceptibility to apoptosis, Fas, FADD, TRADD, Caspase 3 and Caspase 8 are proteins involved in apoptosis[22-25].

Samples were deparaffinized with warm xylol (37ºC), dehydrated in a graded alcohol series and rehydrated with water. Methyl alcohol and H_2O_2 were used for the first endogenous peroxidase blocking, and distilled water and H_2O_2 for the second. The samples were then incubated overnight with the following primary antibodies: anti-PCNA, anti-PTEN, anti-Bcl-2, anti-Fas, anti-FADD, anti-TRADD, anti-Caspase 3 and anti-Caspase 8. Anti-PCNA is a rat monoclonal antibody, clones PC10, DAKO™, DakoCytomation, Hostrup, Denmark. Anti-PTEN is a rat monoclonal antibody, 28H6, NOVOCASTRA™, Newcastle, United Kingdom. Anti-Bcl-2 is a rat monoclonal antibody, clones 124, DAKO™, DakoCytomation, Hostrup, Denmark. Anti-Fas is a rat monoclonal antibody, clones GM30, 1:160, NOVOCASTRA™, Newcastle, United Kingdom. Anti-FADD is a rabbit polyclonal antibody, ab55399, 1:30, ABCAM™, Cambridge, United States of America. Anti-TRADD is a rat monoclonal antibody, clones 18A11, 1:50, DAKO™, DakoCytomation, Hostrup, Denmark. Anti-Capase 3 is a rabbit polyclonal antibody, clone 3CSP03, 1:200, BIOSYSTEMS™, Pleasanton, Canada. Anti-Caspase 8 is a rabbit polyclonal antibody, 1:100, ABR™, Colorado, United States of America. The secondary antibody was incorporated to a dextran polymer for samples incubation for 30 minutes. DAKO ADVANCED™ HRP SYSTEM, DakoCytomation, Inc from CA, USA was used to reveal immunoreactivity, and the slides were counterstained with Mayers' hematoxylin.

Septal cells (fibroblasts, endothelial cells and mononuclear cells) and alveolar cells (pneumocytes) were considered positive when exhibiting the brown nuclear staining (PTEN and PCNA) or perinuclear (Bcl-2), or citoplasmatic staining for Caspase 3, Caspase 8, TRADD and FADD or membrane staining for Fas as expected for each antibody. An Olympus BX50 microscope (Olympus Optical Co, Ltd., Japan) with a 40× objective, was used to examine the slides.

The staining was interpreted as follows: for PCNA, ten high-power fields for each four samples of each case were examined at a magnification of 400×. The total number of positive cells in the high-power fields for the septa and alveoli were counted, and the mean number of positive cells for all four samples was calculated (the diameter of high-power field is 106 micrometers).

For PTEN and Bcl-2, a score was assigned according to the degree of positive staining as well as a pattern of staining. For each of the four samples of each case a degree of positive staining was assigned as follows: 0 for negative samples, 1 for weakly positive staining, and 2 for strongly positive staining. The total score for each case were added to provid the total sum scores for the four samples. The pattern of staining was scored as follows: 1 for focal, 2 for multifocal and 3 for diffuse. The scores for each case were determined by adding the scores for each of the four samples[26].

For Fas, FADD, TRADD, Caspase 3 and Caspase 8 the immunostained slides were observed using an optical microscope Olympus® BX50 (Tokyo, Japan), coupled to a Dino eye video camera enhanced by image analysis software Image Pro Plus™ (Maryland, USA). For each sample, 12 photomicrographs were taken in high-power field [(HPF) = 400×], with a total area of 115,226.1 µm² and with 1024 × 768 pixels each. The positive control HPF photomicrography was chosen as the "mask", which contained adequate levels of positive tissue immunoexpression signal. The mask was then superimposed to the samples photomicrographs. Based on the ideal positive tissue immunoexpression signal obtained from the mask, the image analysis software Image Pro Plus(TM) identified the positive areas in the samples and is able to transform these results into positive tissue immunoexpression area per square micron (µm²). The area in µm² obtained with this method was divided by the constant 115,226.1 µm², which is the total area of the HPF observed, thus generating a percentage value for the positive tissue immunoexpression area for each HPF. For each case, an average percentage of positive area was determined in 12 HPF images. TRADD was not submitted to morphometric analysis because most cases were negative for this protein.

The observer did not have prior knowledge to which group the samples belong.

The data were analyzed using SPSS 20.0 (IBM, São Paulo, SP, Brazil). The tests analysis of variance (Anova), Mann-Whitney,

Kruskal-Wallis, and Fisher were used for statistical analysis. The group "without" CLD was used as the control group for the Dunnett's test. The significance level was set at $p < 0.05$. For the Fisher test the significance level was correct for Bonfarroni ($p < 0.017$). Analysis of covariance (Ancova) was tested using corrected age and birth weight as covariates.

RESULTS

Clinical data

The "classic" CLD group received oxygen therapy for the longest period of time (21.36 days) than the other groups. The clinical profile of the study population is shown in **Tables 2**, **3** and **4**.

Immunohistochemical data

There was a high PCNA tissue immunoexpression, in septal cells in the "classic" form of the disease ($p = 0.057$). PTEN ($p = 0.015$) and Caspase 8 ($p = 0.010$) tissue immunoexpression was greater in the "new" CLD group. FADD expression was greater in the group without CLD ($p < 0.001$). All cases were analyzed immunohistochemically for the proteins, and the results are shown in **Table 5** and **Figure**.

TABLE 2 – Clinical date for the groups

Parameters	Classic CLD	New CLD	Without CLD
Gender			
Female	6	4	11
Male	5	1	5
Birth weight* (g)	1021/289	1340/398	959/358
Gestational age (weeks)*b	32/3.3	33.4/3.1	28.8/2.6
Apgar score first minute* ($n = 29/32$)†	2.6/2	6/3.5	3.5/2.7
Apgar score fifth minute* ($n = 29/32$)†	6.2/2.6	8.4/1.9	5/3.5
Pregnancy hypertension event ($n = 31/32$)†			
Yes	3	3	2
No	8	2	13
Gestational diabetes ($n = 32/32$)†			
Yes	0	1	0
No	11	4	16
Chorioamnionitis ($n = 32/32$)†			
Yes	1	0	2
No	10	5	14
Amniotic sac disruption ($n = 27/32$)†			
Yes	1	1	4
No	9	4	8

*CLD: chronic lung disease; *: mean/standart deviation; †: number of cases; a: Kruskal-Wallis nonparametric test (p < 0.05); b: p = 0.005.*

TABLE 3 – The postnatal clinical factors for the groups

Parameters	Classic CLD ($n = 11$)	New CLD ($n = 5$)	Without CLD ($n = 16$)	p-value
Patent ductus arteriosus				
Yes	10	2	2	0.0003**
No	1	3	14	
Antibiotic therapy				
Yes	10	4	8	> 0.017‡
No	1	1	8	
Surfactant therapy				
Yes	7	0	1	0.0089
No	4	5	15	
Necrotizing enterocolitis				
Yes	7	2	1	0.002‡
No	4	3	15	
Bronchopneumonia				
Yes	6	0	1	0.009‡
No	5	5	15	
Pulmonary hemorrhage				
Yes	3	1	1	> 0.017‡
No	8	4	15	
Pulmonary hypertension				
Yes	2	1	0	> 0.017‡
No	9	4	16	
Intracranial hemorrhage				
Yes	4	2	1	> 0.017‡
No	7	3	15	
Sepsis				
Yes	11	4	3	0.001‡
No	0	1	13	
Corrected gestational age (weeks)*				
	32/3.3	33.4/3.1	28.8/2.6	0.005§
Survival time (days)*				
	23.4/12.3	12.4/5.5	1.4/1.6	< 0.001§
Asphyxia				
Yes	6	1	9	> 0.017‡
No	5	4	7	

*CLD: chronic lung disease; *mean/ standart deviation; **: Pearson test; ‡: Fisher test, p > 0.017 (adjusted for Bonferroni); §: Kruskal-Wallis nonparametric test (p < 0.05).*

TABLE 4 – Use of oxygen by the groups

	Classic CLD	New CLD	Without CLD
Days of mechanical ventilation*	12.90/9.23	3.4/3.91	1.33/1.69
Days of CPAP*	5.6/7.66	4.4/3.2	1.91/1.63
Days of oxygen tent*	3.8	1.8	0
Days of oxygen catheter*	0.27	0.8	0
FiO_2 Max (%)*	95/12.24	85/30	98.75/7.07
PIP Max (mmHg)*	21.16/3.37	20/0	23.18/6.98
PEEP Max (mmHg)*	4.7	5	5.05
Days of oxygen therapy/survival time*	21.36/12.26	10.4/2.79	1.4/1.6

*CLD: chronic lung disease; CPAP: continues positive airway pressure; FiO_2 Max: fraction of inspired oxygen maximum; PIP Max: peak inspiratory pressure maximum; PEEP Max: positive end expiratory pressure maximum; *: mean/ standart deviation.*

TABLE 5 – Immunohistochemical expression of proteins PCNA, PTEN, Bcl-2, Fas, FADD, Caspase 3 and Caspase 8 in each group

Protein	Classic CLD (n = 11) m/SD	New CLD (n = 5) m/SD	Without CLD (n = 16) m/SD	p-value§
PCNA septal (n = 26/32)*	69.1/55.8	61.5/38.1	24.2/40.5	0.057
PCNA alveoli (n = 26/32)*	4.59/3.63	3.25/1.55	3.21/5.55	0.246
Bcl-2 degree	2.18/1.83	1.8/1.79	0.94/1.61	0.236
Bcl-2 pattern	2.18/1.83	2.18/1.83	0.88/1.45	0.196
PTEN degree* (n = 29/32)*	4.67/2	7.5/1	3.63/2.39	0.015
PTEN pattern* (n = 29/32)*	5.78/3.15	8.75/3.95	5/3.86	0.2
Fas[a]	0.032/2289.2	0.039/4650.9	0.030/2483.3	0.967
FADD[a]	0.028/2476.3	0.024/1073.5	0.104/3425.1	< 0.001
Caspase 3[a]	0.012/777.3	0.012/1060.5	0.014/1235	0.993
Caspase 8[a]	0.031/589	0.046/951.7	0.032/766.3	0.010

**: number of positive cells; [a]: immunopositive area (%/SD); §: Kruskal-Wallis nonparametric test (*p *< 0.05).*

PCNA: proliferating cell nuclear antigen; PTEN: phosphatase and tensin homolog; Bcl-2: B cell lymphoma 2; Fas: tumor necrosis factor receptor family member; FAAD: fas-associated protein with death domain; CLD: chronic lung disease; m: mean; SD: standard deviation.

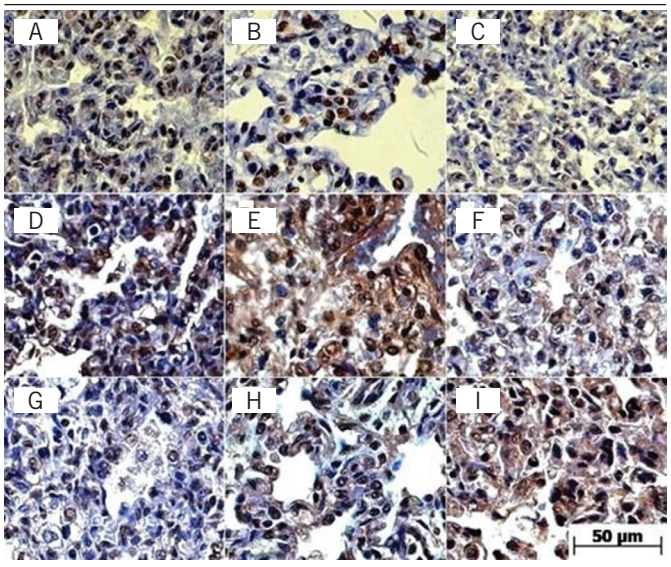

FIGURE – *Immunohistochemical reaction*

A, B and C – immunohistochemical reaction for PTEN. A) "classic" CLD; B) "new" CLD; C) "without" CLD groups cases. There are a few septal and alveoli positive cells in the "without" CLD group, and much more positive septal and alveoli cells in the "new" and the "classic" CLD groups (400×).

D, E and F – Immunohistochemical reaction for Caspase 8. D) "classic" CLD; E) "new" CLD; F) "without" CLD groups cases. There are more positive cells in the "new" CLD group (400×).

G, H and I – Immunohistochemical reaction for FADD. G) "classic" CLD; H) "new" CLD; I) "without" CLD groups. There are more positive cells in the "without" CLD group (400×).

PTEN: phosphatase and tensin homolog; CLD: chronic lung disease; FAAD: fas-associated protein with death domain.

DISCUSSION

BPD is a chronic lung disease, characterized by an impaired lung function due to a reduced final alveolar number and vascular growth. It is the most common sequelae of ventilation premature neonates[27].

Various studies are currently being carried out to elucidate the pathogenesis of this condition. It is known to be multifactorial, with a genetic predisposition, prolonged use of oxygen at high concentrations, insufficient surfactant, exposure to mechanical ventilation (leading to volutrauma, biotrauma and barotrauma) and pre or postnatal infection as the main etiologic factors[8]. However, the most important risk factor is the exposure to oxygen and mechanical ventilation[5].

From the pathological point of view, a injury and repair process, with early alveolar and interstitial inflammation and fibrosis, characterizes "classic" CLD. "New" CLD, on the other hand, as observed by Coalson *et al*. (1999) in baboon lungs, is characterized by alveolar hypoplasia, variable saccular wall fibrosis and minimal airway disease. Coalson *et al*. (1995) also reported decreased alveolarization and internal alveolar surface area. These findings were confirmed by other authors in human, sheep, lamb and rabbit lungs[17-20, 28-30].

During lung development there is a natural equilibrium between apoptosis and cell proliferation[31]. Loss of this equilibrium may result in chronic lung pathologies in newborns as a result of impaired vascular and alveolar growth. The pathophysiology of alveolar hypoplasia, which is present in the "new" CLD, has not been fully elucidated to date. It is known that apoptosis and cell proliferation are implicated in this process and that pulmonary exposure to oxygen and cyclic stretching in mechanical ventilation can trigger changes in the cell-cycle, particularly changes in apoptosis[10]. Fewer alveoli and decreased alveolar perimeter were found in the "new" CLD group compared to the group "without" CLD, confirming impaired alveolar growth in neonates with this pathology.

The number of females is greater than males. Neonates in the "new" CLD group had heavier weight and more mature at birth than in the other two groups (mean weight 1,340 g and mean corrected age 33.4 weeks). The group "without" CLD was more premature at birth (mean 28.8 weeks). The presence of maternal pathology such as hypertensive disorders of pregnancy and gestational diabetes did not influence the incidence of CLD.

From the point of view of infection (chorioamnionitis and membranes rupture for more than 18 hours), comparing the groups, there were no statistically significant differences. However the postnatal infection (bronchopneumonia, necrotizing enterocolitis and sepsis) was greater in the "classic" CLD group,

showing that infection could increase the risk of developing CLD, or because these neonates might be more exposed to oxygen and mechanical ventilation. The presences of pathologies such as pulmonary hemorrhage, pulmonary hypertension and intracranial hemorrhage have no statistically significant differences in the groups.

The length of oxygen therapy use and the time of survival were almost a like. The lungs of premature infants were in different stages of development during the repair/remodeling processes. In the group "without" CLD, the length of time of oxygen therapy and the survival time (mean 1.4 days) were shorter than the other two groups. This could explain the reason why this group has not shown any pathological features of CLD (Table 4).

The "classic" CLD group received oxygen and presented longer survival (21.36 days) compared to the other groups but was, therefore, more exposed to high peak inspiratory pressure and positive expiratory pressure, showing that the oxygen and the agressive mechanical ventilation was involved in CLD development. On the other hand, the "classic" group had more time for cell differentiation, a process that is characteristic for this phase.

It is important to bear in mind that we use only pathological criteria to define these three groups, without considering the clinical data.

Statistically significant differences were found when expression of the proteins was analyzed.

PCNA is a nuclear protein which acts as an accessory factor of deoxyribonucleic acid (DNA) polymerase delta, it is required for DNA replication and repair, and consequently for cell replication[22]. Its expression is increased in cells which are proliferating. If PCNA is reduced or not present in a cell, apoptosis will take place[32]. In this study there was a higher proliferation index, as evidenced by a high PCNA tissue immunoexpression, in septal cells in the "classic" form of the disease compared to the group "without" CLD ($p = 0.057$), showing a statistical tendency (Table 5). The pathological features of the "classic" form showed an obliterative bronchiolitis, an interstitial fibrosis that is compatible with proliferative activity. These findings may be associated with the ability of a pulmonary cell to proliferate in response to mechanical strain[33, 34], knowing that the "classic" form was submitted to mechanical ventilation for more days than the group "without" CLD. Regarding the exposure to oxygen, some studies with cell cultures showed that hyperoxia inhibits cell proliferation[13, 14], while another study say the opposite[35].

There was no difference in proliferation indices between the "new" CLD and "classic" CLD group, and between the "new" CLD and the group "without" CLD. There were no statistically significant differences in the degree of positivity for PCNA in alveolar cells between the groups, which were all positive for this marker as cell proliferation is normally present in the canalicular and saccular stages of lung development, corresponding to the 22nd to 36th weeks of gestation[10].

PTEN is a lipoprotein phosphatase that plays an important role in cell proliferation and apoptosis by negatively regulating the cell-cycle and suppressing growth[36]. PTEN induces cell death by increasing the tumor suppressor activity of p53 and reducing cell proliferation by blocking the phosphoinositol 3 kinase/Akt signaling pathway, with a consequent reduction in Ki67, a protein associated with cell proliferation[23]. The greater the number of cells expressing this protein in a particular tissue, the more likely the tissue would have a higher apoptotic index[36]. In this study, PTEN tissue immunoexpression was greater in the "new" CLD group (degree and pattern score tending to be strong and diffuse staining) than in the group "without" CLD. Caspase 8 immunoexpression was also greater in the "new" CLD group. Caspase 8 is a protein that belongs to the apoptotic pathways. These findings were statistically significant and could indicate that the "new" form of the disease presents more cells susceptible to apoptosis than the group "without" CLD, consistent with the decreased alveolarization observed in this phase of the disease (Figure and Table 5)[6]. Confirming these findings, previous studies in animal models have shown that there is increased apoptosis in lungs exposed to oxygen, which are susceptible to barotrauma, volutrauma and biotrauma[11, 12]. A study by Hargitai in 2001, also found a high apoptotic index in alveolar and bronchial cells in lungs with BPD.

There were no statistically significant differences in PTEN tissue immunoexpression between the "classic" CLD group and the group "without" CLD, compatible with data in literature indicating that "classic" CLD is more associated with inflammatory response than with the apoptosis processe[30].

There was no statistically significant difference in Bcl-2 tissue immunoexpression between the groups. Bcl-2 is an anti-apoptotic protein associated with the regulation of apoptosis, it is of special importance as it is one of the proteins that determine which cells will undergo apoptosis and in which apoptosis it will be inhibited[24].

In the present study, Bcl-2 expression was higher in "classic" CLD than in the group "without" CLD (Table 5), but this finding has not been statistically significant. However, this result might be related to the better long-term survival of the group with "classic" CLD, which allowed more time for cell differentiation and consequently led to increased Bcl-2 expression, indicating higher resistance to apoptosis.

Fas is a protein that belongs to the subgroup of tumor necrosis factor receptor (TNF-R), this protein can trigger apoptosis. Stimulation of the Fas receptor results in its trimerization and recruitment of two key signaling proteins,

the adapter protein Fas-associated death domain, FADD, and the initiator cysteine protease Caspase 8. Subsequent activation of the effector caspases through mitochondria dependent or independent pathways results in activation of Caspase 3, the key effector caspase. Activated Caspase 3 cleaves a variety of substrates, including DNA repair enzymes, cellular and nuclear structural proteins, endonucleases, and many other cellular constituents, culminating in effective cell death[37-39].

FADD is an adaptor molecule that mediates cell apoptotic signals. It can allow recruiting Caspase 8 or Caspase 10 to the activated Fas receptor. The resulting aggregate called the death-inducing signaling complex (DISC) performs Caspase 8 proteolytic activation. Active Caspase 8 initiates the subsequent cascade of caspases mediating apoptosis, and then FADD binding to Fas receptor is degraded. The antibody used in this study can bind Fas into FADD portion, in free FADD. Thus in this study, FADD expression was higher in the group without CLD. This may indicate that apoptosis is lower or did not happen in the group without CLD (FADD-Fas was not degraded) comparing to other groups (Figure)[40].

For immunoexpression of the other proteins involved in cell apoptosis (Fas and Caspase 3) there were no statistically significant differences in the groups. In the literature, the function of Caspase 3 is not clear, Caspase 3 could be involved in the pathophisiology of oxygen and ventilation inducing apoptosis[10].

CONCLUSION

Apoptosis was involved in the pathophysiological of CLD. The process of lung tissue lesion appears to be related to an imbalance between inflammatory response, apoptosis and cell proliferation that affects alveolar formation and pulmonary vascular growth[2-4]. Increased apoptosis ("new" form) and cell proliferation in lungs with CLD were observed in this study.

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ETHICAL CONDUCT OF RESEARCH

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. The ethics review board of the HC-UFPR reviewed and approved the study (Register number 1099.138/2005-08; approval 30 August 20).

RESUMO

Introdução: A fisiopatologia da doença pulmonar crônica, clinicamente conhecida como displasia broncopulmonar (DBP), ainda é incerta. Acredita-se que mecanismos de proteção, como liberação de mediadores inflamatórios e ativação de processos apoptóticos e/ou proliferativos, são acionados no tecido pulmonar de prematuros na tentativa de reparar os danos teciduais causados pela exposição ao oxigênio e à ventilação mecânica. Objetivo: Avaliar a existência de apoptose e proliferação celular em pulmões de neonatos prematuros com DBP, expostos ao oxigênio e/ou à ventilação mecânica, por meio do estudo da expressão das proteínas: antígeno nuclear de proliferação celular (PCNA), homólogo da fosfatase e tensina (PTEN), linfoma de células B 2 (Bcl-2), membro da família de receptor do fator de necrose tumoral (Fas), proteína de domínio de morte associada ao Fas (FADD), proteína do domínio de morte associada ao receptor do fator de necrose tumoral (TRADD), Caspase 3 e Caspase 8. Material e método: Foram analisadas 32 autópsias de recém-nascidos, com idade gestacional inferior a 34 semanas, expostos ao oxigênio. O estudo foi dividido em três grupos: DBP "clássica", DBP "nova" e "sem" DBP; realizou-se estudo imuno-histoquímico. Resultados e discussão: Um índice de proliferação mais elevado foi observado nos recém-nascidos com DBP, sugerindo que o maior tempo de exposição à ventilação mecânica pode estimular a proliferação celular. A expressão das proteínas PTEN e Caspase 8 foram maiores no grupo da DBP "nova" em relação ao grupo sem DBP, indicando que a DBP "nova" é mais suscetível à apoptose. Conclusão: A apoptose e a proliferação celular estão envolvidas na fisiopatologia da DBP, sendo a apoptose mais evidente no grupo com DBP "nova".

Unitermos: *apoptose; biomarcadores farmacológicos; proliferação de células; displasia broncopulmonar; neonatologia.*

REFERENCES

1. Pedreira PR, Garcia-Prieto E, Albaiceta GM, Taboada YF. Respuesta inflamatória y apoptosis em La lesión pulmonar aguda. Med Intensiva. 2006; 30: 268-75.

2. Speer CP. Inflammation and bronchopulmonary dysplasia. Semin Neonatol. 2003; 8: 29-38.

3. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semim Fetal Neonatal Med. 2006; 11: 354-62.

4. Turato G, Zuin R, Saetta M. Pathogenesis and pathology of COPD. Respiration. 2001; 68: 117-28.

5. Jobe H, Bancalari A, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001; 163: 1723-9.

6. Shawn K, Conway A, Conway S. Aberrant signaling pathways of the lung mesenchyme and their contributions to the pathogenesis of bronchopulmonary dysplasia. Birth Defects Res A Clin Mol Teratol. 2012; 94: 3-15.

7. Tapia JL, Agost D, Alegria A, et al. Bronchopulmonary dysplasia: incidence, risk factors and resource utilization in a population of South American very low birth weight infants. J Pediatr. 2006; 82: 15-20.

8. Bhandari A, Bhandari V. Bronchopulmonary dysplasia an update. Indian J Pediatr. 2007; 74: 73-7.

9. Shaw GM, O'Brodovich HM. Progress in understanding the genetics of bronchopulmonary dysplasia. Seminars in Perinatology. 2013; 37: 85-93.

10. May M, Ströbel P, Preisshofen T, Seidenspinner S, Marx A, Speer CP. Apoptosis and proliferation in lungs of ventilated and oxygen-treated preterm infants. Eur Respir J. 2004; 23: 113-21.

11. Barazzone C, Horowitz S, Donati YR, Rodriguez I, Piguet PF. Oxygen toxicity in mouse lung: pathways to cell death. Am J Respir Cell Mol Biol. 1998; 19: 573-81.

12. McGrath-Morrow SA, Stahl J. Apoptosis in neonatal murine lung exposed to hyperoxia. Am J Respir Cell Mol Biol. 2001; 25: 150-5.

13. Hussain N, Wu F, Christian C, Kresch MJ. Hyperoxia inhibits fetal rat lung fibroblast proliferation and expression of procollagens. Am J Physiol. 1997; 273: 726-32.

14. Rancourt RC, Staversky RJ, Keng PC, O'Reilly MA. Hyperoxia inhibits proliferation of Mv1Lu epithelial cells independent of TGF-β signaling. Am J Physiol. 1999; 277: 1172-8.

15. Mokres LM, Parai K, Hilgendorff A, et al. Prolonged mechanical ventilation with air induces apoptosis and causes failure of alveolar septation and angiogenesis in lungs of newborn mice. Am J Physiol Lung Cell Mol Physiol. 2010; 298: 23-35.

16. Hargitai B, Szabó V, Hajdú J, et al. Apoptosis in various organs of preterm infants: histopathologic study of lung, kidney, liver, and brain of ventilated infants. Pediatr Res. 2001; 50: 110-4.

17. Okamoto CT, Silva LLG, Noronha L, Bahr JA. Análises histopatológica e morfométrica no diagnóstico da "nova" displasia broncopulmonar e comparação clinicopatológica com a forma clássica da doença. J Bras Patol Med Lab. 2009; 45: 155.

18. Coalson JJ, Winter V, Lemos RA. Decreased alveolarization in baboon survivors with bronchopulmonary dysplasia. Am J Respir Crit Care Med. 1995; 152: 640-6.

19. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med. 1999; 160: 1333-46.

20. Mascaretti RS, Mataloun MMGB, Gibelli MACB, et al. Preterm rabbits exposed to prolonged hyperoxias as a model for the study of bronchopulmonary dysplasia. Pediatr Res. 2003; 53: 436.

21. Cronin M, Pho M, Dutta D, et al. Measurement of gene expression in archival paraffin-embedded tissues: development and performance of 92- gene reverse transcriptase-polymerase chain reaction assay. Am J Pathol. 2004; 164: 35-42.

22. Gary R, Kim K, Cornelius HL, Park MS, Matsumoto Y. Proliferating cell nuclear antigen facilitates excision in long-patch base excision repair. J Biol Chem. 1998; 274: 4354-63.

23. Weng LP, Brown JL, Eng C. PTEN induces apoptosis and cell cycle arrest through phosphoinositol-3-kinase/Akt-dependent and independent pathways. Hum Mol Genet. 2001; 10: 237-42.

24. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science. 1998; 281: 1322-6.

25. De Paepe ME, Gundavarapu S, Tantravahi U, et al. Fas-ligand-induced apoptosis of respiratory epithelial cells causes disruption of postcanalicular alveolar development. Am J Pathol. 2008; 173: 42-56.

26. Allred DC, Harvey JM, Berardo M, Clarck GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol. 1998; 11: 144-68.

27. Sánchez Luna M, Moreno Hernando J, Bolet Mussons F, et al. Displasia broncopulmonar: definiciones y clasificación. An Pediatr. 2013; 79: 4.

28. Grisafi D, Tassone E, Dedja A, et al. L-citrulline prevents alveolar and vascular derangement in a rat model of moderate hyperoxia-induced lung injury. Lung. 2012; 190: 419-30.

29. Grisafi D, Pozzobon M, Dedja A, et al. Human amniotic fluid stem cells protect rat lungs exposed to moderate hyperoxia. Pediatr Pulmonol. 2013; 48: 1070-80.

30. Rebello CM, Mascaretti RS. A nova displasia broncopulmonar. In: Manual de atualizações em Neonatologia. Rio de Janeiro: Panmericana; 2004. p. 87-132.

31. Weaver TE, Whitsett JA. Function and regulation of expression of pulmonary surfactant-associated proteins. Biochem J. 1991; 15: 249-64.

32. Paunesku T, Mittal S, Protić M, et al. Proliferating cell nuclear antigen (PCNA): ringmaster of the genome. Int J Radiat Biol. 2001; 77: 1007-21.

33. Maniscalco WM, Watkins RH, O'Reilly MA, Shea CP. Increased epithelial cell proliferation with chronic lung disease. Am J Physiol Lung Cell Mol Physiol. 2002; 283: 991-1001.

34. Chess PR, Toia L, Finkelstein JN. Mechanical strain-induced proliferation and signaling in pulmonary epithelial H 441 cells. Am J Physiol Lung Cell Mol Physiol. 2000; 279: 43-51.

35. Gao Y, Xue XD, Li JY, Wang N. Expression and roles of CDK4 and p21 in lung tissues of premature rats with hyperoxia-induced chronic lung disease. CJCP. 2007; 9: 595-600.

36. Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. Cell. 2000; 100(4): 387-90.

37. Nagata S. Fas ligand-induced apoptosis. Annu Rev Genet. 1999; 33: 29-55.

38. Sharma K, Wang RX, Zhang LY, et al. Death the Fas way: regulation and pathophysiology of CD95 and its ligand. Pharmacol Ther. 2000; 88: 333-47.

39. Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res. 2000; 256: 58-66.

40. Anti-FADD ab55399, ABCAM™, Cambridge, United States of America.

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4.2 ARTIGO 2

QUANTITATIVE ANALYSIS OF INFLAMMATORY AND ADHESION MOLECULES IN LUNGS OF NEONATES WITH CHRONIC LUNG DISEASE (BRONCHOPULMONARY DYSPLASIA) RECEIVING MECHANICAL VENTILATION.

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Quantitative analysis of inflammatory and adhesion molecules in lungs of neonates with chronic lung disease (bronchopulmonary dysplasia) receiving mechanical ventilation

Análise quantitativa de moléculas inflamatórias e de adesão em pulmões de neonatos com doença pulmonar crônica (displasia broncopulmonar) submetidos à ventilação mecânica

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ABSTRACT

Introduction: Chronic lung disease (CLD), clinically known as bronchopulmonary dysplasia (BPD), is a major cause of morbidity in premature newborn and were submitted to oxygen therapy. **Objective:** Immunohistochemical identification of inflammatory molecules in the lung tissue of premature neonates that died with CLD. **Methods:** Immunohistochemical analysis of 51 samples of premature newborn lungs – grouped in: without CLD, "classic" CLD and "new" CLD. **Results:** Neutrophil influx and the number of CD4+ and CD45RO+ cells were higher in the "classic" CLD group ($p < 0.001$). **Conclusion:** Our findings suggest that the inflammatory response is mediated by neutrophils and CD45RO+ and CD4+ T lymphocytes in the "classic" CLD.

Key words: bronchopulmonary dysplasia; immunochemistry; premature birth; oxygen therapy; neonatology.

INTRODUCTION

Chronic lung disease (CLD) or, clinically, bronchopulmonary dysplasia (BPD) is a chronic neonatal pulmonary disorder and a major cause of morbidity affecting premature newborn[1-3]. The classic presentation of this disorder was described in 1960 as a consequence of respiratory distress syndrome, the use of higher concentrations of inspired oxygen and aggressive mechanical ventilation produced characteristic radiological changes. Histological findings of "classic" CLD are emphysema, atelectasis, fibrosis, squamous metaplasia and smooth muscle hypertrophy in the airways and pulmonary vasculature[4].

The use of prenatal corticosteroids therapy, exogenous surfactant and technological advances in mechanical ventilation have resulted in a significant increase in the survival rates of newborn with very low birth weight; however, the incidence of CLD has not changed as expected[4-7]. Instead, another type of CLD, known as "new" CLD emerged, particularly in newborn with less than 30 weeks of gestational age who continued to require supplemental oxygen after 28 days of age[3-6].

In 2001, a consensus suggested that the clinical criteria for the diagnosis of BPD/CLD ("new" or "classic") in a preterm newborn must depend on the use of respiratory support at 28 days of postnatal life or 36 weeks postmenstrual age[3, 6-9]. Although the pathological criteria for "classic" CLD are characterized by an intense inflammatory response and septal fibrosis, in the "new" CLD there is decreased alveolar septation and abnormal pulmonary vascular development compatible with impaired pulmonary growth. Septal fibrosis, inflammatory response and interstitial edema are rarely observed. The alterations in alveolar formation may be very subtle and sometimes not detectable in routine morphological tests.

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The characteristics of the "new" CLD may be more related to morphometric data than to the morphological aspects[10-14].

Over the last 10 years, the pathological basis of CLD have predominantly been associated with the following two processes: inflammation and arrest of lung development[2, 4, 5]. The inflammatory response in CLD is exacerbated by mechanical ventilation and exposure to supplemental oxygen. Several studies seem to indicate that neutrophils prevail in the earliest stages of CLD, followed by an abundance of macrophages and other inflammatory cells, increased pulmonary vascular permeability and damage to endothelial and epithelial cells, typically leading to apoptosis[15-21].

The objectives of this study were to investigate the subtypes of inflammatory cells and adhesion molecules, using immunohistochemistry in lung samples of preterm newborn receiving mechanical ventilation (less than 34 weeks gestational age) and to correlate the immunoexpression of these biomarkers with the presence or absence of CLD.

METHODS

Sample selection

The sample consisted of 51 specimens of preterm newborn lung autopsies performed between 1994 and 2005 (after the pre-surfactant era); from the archives of the Department of Pathology Medicine of Universidade Federal do Paraná (UFPR). The research ethics board of the Hospital das Clínicas (HC)-UFPR reviewed and approved the study (registration number 1099.138/2005-08; approved on August 30, 2005). The following inclusion criteria were used: complete autopsy; absence of major malformations; absence of meconium aspiration; less than 34 weeks of gestational age and use of oxygen therapy for at least 2 hours. The samples that were unsuitable for testing or for which the medical records were not available were excluded. The medical records were analyzed to collect data related to antenatal risk factor for CLD including gender, gestational age, birth weight, maternal age, number of pregnancies, prenatal care, hypertensive event in pregnancy, gestacional diabetes, chorioamnionitis, premature amniotic sac disruption, type of birth and twin pregnancy. Additionally, other potential postnatal risk factors for CLD include patent ductus arteriosus, cardiopulmonary resuscitation, Apgar score at the first e fifth minute, antibiotic therapy, surfactant replacement therapy, necrotizing enterocolitis, bronchopneumonia, pulmonary hemorrhage, pulmonary hypertension, choking, intracranial hemorrhage, pneumothorax, sepsis and corrected gestational postnatal age were recorded. Survival time and cause of death were also recorded[10].

Morphometric analysis

The slides of the 51 patients were stained with hematoxylin and eosin (HE) and divided into the following three groups: 1) histopathological findings of "classic" CLD (n = 11); 2) histopathological and morphometric findings of "new" CLD (n = 5); and 3) "without" histopathological and morphometric criteria for CLD (n = 35). Since the alterations in alveolar formation may be very subtle, the samples of groups 2 and 3 were analyzed only to confirm the existence of the morphometric criteria of "new" CLD in group 2[9, 10, 22, 23].

The digital images of 10 nonadjacent fields (200× = 106 micrometers in diameter) in slides of the pulmonary parenchyma from the 40 cases (group 2 and 3) were captured using Image Pro Plus software™ (Rockville, MD, USA). It measures the alveolar perimeter in micrometers and the number of alveoli per field[10, 22, 23]. The alveoli in each of the 10 digitalized fields were measured. Means and standard deviations for each case were calculated. One blinded observer performed these analyses[10, 23].

Immunohistochemical staining

Paraffin blocks of the studied cases (n = 51) were used to construct the tissue microarrays (TMAs)[23]. Lung samples from all the patients were inserted into multi-sample paraffin blocks (TMAs), with four samples of each case and 3 mm diameter for each sample (analyzed area = 28.26 mm^2). Histological sections of TMA blocks were used for immunohistochemical analysis (immunoperoxidase staining). The protocol for the immunoperoxidase assay was standardized with positive and negative controls. The cases were tested with the following antibodies: anti-CD14 (clone 7, mouse monoclonal); anti-CD4 (clone 4B12, kappa 1, mouse monoclonal); anti-CD8 (clone 1A5, mouse monoclonal); anti-CD74 antibody (clone LN-2, mouse monoclonal antibody); anti-CD25 antibody (clone 4C9, mouse monoclonal antibody, alpha subunit of the interleukin-2 [IL-2] receptor); anti-CD54 or anti-intercellular adhesion molecule-1 (ICAM-1) antibody (clone 23G12, mouse monoclonal antibody); and anti-CD106 or anti-vascular cell adhesion molecule (VCAM) antibody (clone 1.4C3, mouse monoclonal antibody), all from Novocastra™ (Leica Biosystems, Buffalo Grove, USA); and anti-CD45RO (clone UCHL, kappa, mouse monoclonal antibody) and anti-CD20 (clone IF5; mouse monoclonal antibody) from Dako (Denmark A/S; Glostrup, Denmark)[21, 23, 24].

Staining was interpreted as follows: for anti-CD4, CD8, CD14, CD20, CD25, CD74 and CD45RO antibodies, the number of positive cells in four random high-power fields (400× = 53 micrometers in diameter) were counted for each of the four samples of each case[23, 24]. The cells that had a characteristic membrane-staining pattern when observed under Olympus BX50 microscope (Olympus Optical Co, Ltd., Japan) and which were located in the alveolar septum or intra-alveolar space were considered positive. The cells in the intravascular space or in regions that usually contain peribronchial lymphoid aggregates were disregarded[24].

For the anti-ICAM-1 (CD54) and anti-VCAM (CD106) antibodies, the slides were assigned a positive score (0 = negative; 1 = weak positive; 2 = strong positive), and the positive score for each case was obtained by the sum of the scores for each of the four samples for this case. The stains were considered positive if they were observed in alveolar septa vessels and in macrophages/monocytes in the septa and alveolar lumina. The positivity of these reactions was assessed in all fields of the four samples of each case using the Olympus BX50 microscope (Olympus Optical Co, Ltd., Japan) at 400× magnification[23].

Statistical methods

Data were analyzed using SPSS 20.0 (IBM, São Paulo, SP, Brazil). Kruskall-Wallis and nonparametric Mann-Whitney test were used for testing quantitative variables. Categorical variables were analyzed using Pearson's chi-square test. Analysis of covariance (Ancova) was used for gestational age adjustment. Statistical significance was accepted for p values < 0.05.

RESULTS

Histopathological and morphometric data

In group 1 ("classic" CLD), the predominant histopathological characteristics were hyaline membranes and interstitial fibrosis[10].

Cases with "new" CLD (group 2) had minimal histopathological alterations, including subtle septal edema. The alveolar changes caused by insufflation and a very subtle inflammatory response were present in these cases[10]. Since the histological findings for the group with "new" CLD were often indistinguishable from those for the group "without" CLD (group 3), a morphometric analysis was required, which showed a decreased in the number and in the perimeter of the alveoli in the "new" CLD group, p = 0.033 and 0.018, respectively (**Table 1** and **Figure**).

TABLE 1 – The number of alveoli and the alveolar perimeter (in micrometers) in the "without" CLD and "new" CLD groups

Parameters	New CLD* (n = 5)	Without CLD* (n = 35)	p value
Number of alveoli	40.9 ± 15.1	57.7 ± 16.2	0.033§
Alveolar perimeter‡	481.5 ± 182.4	611.3 ± 206.2	0.018†

**: average ± standard deviation; §: Mann-Whitney non-parametric test (p < 0.05); ‡: square micrometers; †: Ancova (p < 0.05) (gestational age adjustment).*

CLD: chronic lung disease; Ancova: analysis of covariance.

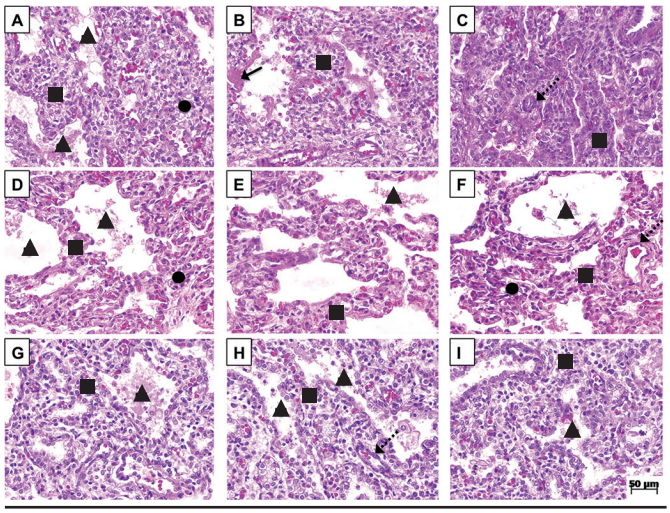

FIGURE – *Photomicrograph of groups*

A, B and C) photomicrograph of group 1 (classic BPD), here we observe areas of hyperinflation (▲) interspersed with areas of atelectasis (●) associated with marked fibrotic septal thickening and chronic inflammatory process (■). In other areas hyaline membranes (arrow) are observed as well as septal arterioles muscle layer thickening (dashed arrow); D, E and F) photomicrograph of group 2 (new BPD), here we observe areas of hyperinflation (▲) interspersed with areas of atelectasis (●) associated with minimal septal thickening with mild edema and mild chronic inflammatory process (■). There are no hyaline membranes and the septal arterioles muscle layer thickening (dashed arrow) is very mild; G, H and I) photomicrograph of group 3 (without BPD), in which we observed few areas of hyperinflation (▲) associated with discrete septal thickening with mild edema (■). There are no hyaline membranes and septal arterioles muscle layer thickening (dashed arrow) are minimal. The alterations found in group 3 are more associated with pulmonary immaturity than neonatal hypoxic injury.

BPD: bronchopulmonary dysplasia.

Clinical data

The "classic" CLD group received oxygen therapy for the longest period of time (p < 0.0001). The risk factors/therapeutic profile of the study sample, including oxygen therapy parameters, gender, gestational age, maternal age and birth weight are shown in **Table 2**, **3** and **4**.

TABLE 2 – Clinical data for the three groups

Parameters	Total samples (n = 51)	Classic CLD (n = 11)	New CLD (n = 5)	Without CLD (n = 35)	p value
Gender†					
Female	33	6	4	23	
Male	18	5	1	12	- -
Gestational age (weeks)	27 (28.9 ± 2.6)*	27 (27-34.5)‡	33 (27-34.5)‡	27 (25-33)‡	0.04§
Birth weight (g) (n = 49/51)	1760 (972.9 ± 342.7)*	1021 (685-1650) ‡	1340 (900-1760)‡	870 (515-1695)‡	0.03§
1-minute Apgar score (n = 45/51)	3 (3.5 ± 2.7)*	2.6 (1-5)‡	6 (1-8)‡	3.4 (1-8)‡	- -
5-minute Apgar score (n = 47/51)	7 (5.9 ± 2.9)*	6.2 (2-9)‡	8.4 (5-10)‡	5.5 (0-10)‡	- -
Maternal age (years) (n = 48/51)	25 (25 ± 6.4)*	26.2 (16-36)‡	29.8 (18-36)‡	24.1 (15-37)‡	- -
Number of pregnancies (n = 50/51)†					
1 or 2	30	6	2	22	
≥ 2	20	5	3	12	- -
Follow-up prenatal visits (n = 50/51)†					
Yes	27	7	4	16	
No	23	4	1	18	- -
Hypertensive event in pregnancy (n = 50/51)†					
Yes	12	3	3	5	
No	38	8	2	29	0.01§§
Gestational diabetes (n = 51/51)†					
Yes	1	0	1	0	
No	50	11	4	35	- -
Chorioamnionitis (n = 49/51)†					
Yes	5	1	0	4	
No	44	10	5	29	- -
Premature amniotic sac disruption (n = 45/51)†					
Yes	15	1	1	13	
No	30	9	4	17	- -
Type of birth (n = 51/51)†					
Normal delivery	29	6	2	21	
Cesarean delivery	22	5	3	14	- -
Twin pregnancy (n = 51/51)†					
Yes	12	5	1	6	
No	39	6	4	29	- -

*CLD: chronic lung disease; *: median (average ± standard deviation); †: number of cases; ‡: median (min-max); §: Kruskal-Wallis non-parametric test (p < 0.05); §§: Pearson correlation; - -: not statistically significant data.*

Immunohistochemical data

The neutrophil influx was much higher in "classic" CLD group than in "new" CLD or the "without" CLD groups; this difference was statistically significant ($p < 0.001$) (**Table 5**).

There were more CD25+ lymphocytes in the group with "new" CLD than in the other groups ($p = 0.0400$). There were more CD74+ cells and CD4+ T lymphocytes in "classic" CLD group ($p = 0.0500$ and 0.0160, respectively). The CD45RO+ T cell count was significantly higher in "classic" CLD group than in "without" CLD and "new" CLD groups ($p = 0.0004$). The CD14+ cell count was higher in the alveolar septum in "new" CLD group than in "classic" CLD or "without" CLD groups, with a trend toward significance ($p = 0.0523$), as shown in Table 5. There was no statistically significant difference between the groups in terms of CD20+ cell and CD8+T cell counts.

Whereas ICAM-1 expression was higher in the "new" CLD group than in the other groups ($p = 0.0200$), there were no statistically significant differences in VCAM expression between the groups.

DISCUSSION

The pathogenesis of BPD/CLD is complex and remains unclear despite numerous studies on the subject. The lungs of very premature neonates have immature distal airways with a thick blood-air barrier and a small area for gas exchange. Furthermore, they are deficient in surfactant because of the predominance of undifferentiated epithelial cells and the scarcity of type II alveolar cells[25].

Several other factors act additively and synergistically, triggering the inflammatory response and lung injury. These factors include oxygen supplementation, mechanical ventilation, prenatal and postnatal infections, and persistent patency of the

TABLE 3 – The postnatal factors for the three groups

Parameters	Total samples (n = 51)	Classic CLD (n = 11)	New CLD (n = 5)	Without CLD (n = 35)	p value
Patent ductus arteriosus†					
Yes	15	10	2	3	- -
No	36	1	3	32	
Cardiopulmonary resuscitation†					
Yes	40	8	3	29	- -
No	11	3	2	6	
Antibiotic therapy†					
Yes	33	10	4	19	- -
No	18	1	1	16	
Surfactant replacement therapy†					
Yes	15	7	0	8	- -
No	36	4	5	27	
Necrotizing enterocolitis†					
Yes	11	7	2	2	0.0001§§
No	40	4	3	33	
Bronchopneumonia†					
Yes	12	6	0	6	0.0165§§
No	39	5	5	29	
Pulmonary hemorrhage†					
Yes	9	3	1	5	- -
No	42	8	4	30	
Pulmonary hypertension†					
Yes	7	2	1	4	- -
No	44	9	4	31	
Choking†					
Yes	28	6	1	21	- -
No	23	5	4	14	
Intracranial hemorrhage†					
Yes	11	4	2	5	- -
No	40	7	3	30	
Pneumothorax†					
Yes	3	1	0	2	- -
No	48	10	5	33	
Sepsis†					
Yes	24	11	4	9	0.0001§§
No	27	0	1	26	
Corrected gestational age (weeks)*	29 (29.7 ± 3.2)*	32 (29-39)‡	34 (28-36)‡	27 (25-33)‡	0.0002§
Survival time (days)	3 (7.6 ± 10.7)*	23.4 (11-50)‡	12.4 (8-21)‡	1 (0-6)‡	0.0001§
Cause of death†					
Sepsis	24	11	4	9	
Intracranial hemorrhage	4	0	1	3	
Perinatal asphyxia	11	0	0	11	- -
Others	22	0	0	22	

*CLD: chronic lung disease; *: median (average ± standard deviation); ‡: median (min-max); †: number of cases; §: Kruskal-Wallis non-parametric test (p < 0.05); §§: Pearson correlation; - -: not statistically significant data.*

TABLE 4 – The parameters of oxygen therapy for the three groups

Parameters	Classic CLD‡ (n = 11)	New CLD‡ (n = 5)	Without CLD‡ (n = 35)	p value
Mechanical ventilation (days)	12.9 (1-29)/1	3.4 (1-9)/1	1.4 (0.02-6)/2	- -
CPAP (days)	5.6 (0.04-19)/1	4.40 (1-8)/0	1.91 (0.04-5)/25	- -
Oxygen tent (days)	3.8 (1-30)/5	1.8 (1-5)/1	-/35	- -
Catheter oxygen therapy†				- -
Yes	1	1	0	
No	10	4	35	
Oxygen therapy (days)	21.36 (11-50)	10.40 (8-15)	1 (0.02-6)	< 0.001§
FiO_2max (%)	95 (40-100)	85 (40-100)	98.57 (60-100)	- -
PIPmax (mmHg)	21.16 (18-30)	20 (20-20)	22.93 (17-35)	- -
PEEPmax (mmHg)	4.78 (4-5)	5 (5-5)	5 (5-6)	- -

CLD: chronic lung disease; ‡: mean (max-min)/median; †: number of cases; CPAP: continuous positive airway pressure; FiO_2max: maximum fraction of inspired oxygen; PIPmax: maximum peak inspiratory pressure; PEEPmax: maximum positive end-expiratory pressure; §: statistical analysis not significant (p > 0.017); Fisher test (Bonferroni correction).

TABLE 5 – The median neutrophils count, the positive cells per high power field for each marker and the mean VCAM and ICAM-1 positive scores for each group

Marker	Classic CLD ($n = 11$)	New CLD ($n = 5$)	Without CLD ($n = 33$)	p value[§]
Neutrophils count	3.11	1.49	0.59	< 0.001
CD20+	1.5	2.12	1.25	0.938
CD25+	0.3214	0.95	0.3839	0.04
CD45RO+	5.25	1.36	2	0.0004
CD14 (alveolar septum)	3	341.5	15	0.0523
CD14 (alveolar space)	2	61	11	0.138
CD4+	10.887	2.766	2.86	0.016
CD8+	0.472	2.5	3.991	0.17
CD74+	12.25	9.625	7.187	0.05
ICAM	0.106	0.6	0.4292	0.02
VCAM	0	0.2	1	0.076

*VCAM: vascular cell adhesion molecule; ICAM-1: intercellular adhesion molecule-1; CLD: chronic lung disease; [§]: Kruskal-Wallis non-parametric test (*p *< 0.05);* p *value is between classic and without CLD and new and without CLD.*

arterial duct, malnutrition, vitamin A deficiency and genetic factors. Inflammation, however, is the common final pathway for the factors that cause lung injury in CLD[26].

In this study, the "new" CLD group was distinguished from the "without" CLD group by morphometric analysis. This analysis revealed statistically significant differences between the two groups, with a decrease in the number and the perimeter of the alveoli in the "new" CLD group[10, 12-14, 22].

The clinical, histopathological and morphometric patterns of CLD have changed with the emergence of the concept of underdeveloped distal airways. The histopathological findings for "new" CLD were recently described and were observed in our study[10, 12]. As observed in this study, although the clinical criteria for the diagnosis of CLD should depend on respiratory support at 28 days of postnatal life, the pathological changes seem to precede this clinical concept based on the pathological changes found in the infants with 7-28 days of oxygen therapy.

The imbalance between pro-inflammatory and anti-inflammatory activity and its close relationship with cellular apoptosis and proliferation may affect alveolar formation and pulmonary vascular growth, eventually leading to pulmonary hypoplasia[19, 26, 27].

Furthermore, neutrophils may produce recruiting factors for other leukocytes, such as interleukin-8 (IL-8), as well as for tumor necrosis factor (TNF), which is responsible for prolonging neutrophil survival by inhibiting the activation of the apoptotic cascade. Some authors have shown that there is increased evidence of CD4+ and CD8+ T lymphocyte involvement in the mobilization and maintenance of neutrophils at the injury site, which may be a key factor, particularly in CLD, bronchial hyper-reactivity and bronchiectasis[27-30].

Our findings show that the neutrophils influx was higher in the "classic" CLD group than in the "without" CLD and "new" CLD" groups ($p < 0.001$). Furthermore, the number of CD4+ T lymphocytes was higher in the "classic" CLD group than in the "new" CLD group ($p = 0.016$), corroborating the hypothesis that the inflammatory response is more intense in the "classic" CLD form[20, 28, 29, 31].

The physiopathological relationship between the accumulation of neutrophils and the presence of CD4+ T lymphocytes has not been fully clarified. There is some evidence that interleukin-17 (IL-17) plays a significant role in the action orchestrated by these two cells[31-33].

IL-17 released by the CD45RO+ T lymphocytes may activate T cells, which act as mediators in the increased neutrophil recruitment and activation in the damaged tissues, particularly the lung tissue[34, 35]. This finding was confirmed in studies in which azithromycin was used as an inhibitor of IL-17 secretion, and a reduction in tissue damage was observed[35-38].

In our study, the CD45RO+ T lymphocyte count was higher in the "classic" CLD than in the "new" CLD and "without" CLD groups ($p = 0.0004$). It was followed by an increased neutrophil count in the same group ($p < 0.001$), corroborating the hypothesis of an initial inflammatory response mediated by neutrophils and maintained by T lymphocytes in this form of CLD[20, 28, 29, 31].

CD14 is a glycoprotein expressed on the myelomonocytic lineage cells, including monocytes, macrophages and Langerhans cells, and CD14 acts as an opsonin receptor to promote the release of pro-inflammatory cytokines, particularly those associated with Toll-like receptors (TLR)[39, 40]. In our study, the number of CD14+ cells was higher in the "new" CLD group than in the "classic" CLD or the "without" CLD groups, particularly in the alveolar

septum, for which the results showed a trend toward a statistically significant difference ($p = 0.0523$). Some studies have shown that CD14 expression in peripheral blood is lower in premature infants than in term neonates[41-43].

The anti-CD74 antibody detects a major histocompatibility complex (MHC) class II receptor on the macrophage membrane that is involved in macrophage migration inhibitory factor (MIF) induction. CD74+ cells are usually macrophages, histocytes or monocytes, as well as type II pneumocytes, and they are correlated with the induction of type II pneumocyte proliferation in alveolar epithelium repair[44].

Furthermore, Kevin and Bandhari found elevated levels of CD74+ cells and MIF in the lungs of premature rats with respiratory distress syndrome and in tracheal aspirate from premature neonates. Both factors are related to a slight probability of developing CLD. This finding may be explained by the role played by CD74+ cells in pneumocyte proliferation, exacerbation of angiogenesis, increase in leukocyte migration, and therefore the maintenance of inflammatory response[45].

We found more CD74+ cells in the "classic" CLD group than in the other groups, which may be explained by the fact that during the process of alveolar damage that is observed in "classic" CLD, there is an extensive damage to type I pneumocytes, which are replaced by hyaline membranes. The acute phase is followed by the subacute phase, in which the area is cleaned by abundant macrophages and the alveolar lining is regenerated by type II pneumocyte proliferation. The macrophages and type II pneumocytes have the MIF receptor (CD74), and therefore they reacted with the anti-CD74 antibody used in this study[45].

CD25+ T-cells were more abundant in the "new" CLD group. This finding can be explained by the fact that regulatory T cells modulate the inflammatory response, resulting in less inflammation and fibrosis in this form of the disease[46].

Neutrophil migration across the endothelium and epithelial cell barrier depends on the ICAM-1 adhesion receptor, a molecule stimulated in inflammatory disorders that can be induced by cytokines and detected in soluble form in plasma, which has been proposed as a marker of inflammatory activity. The endothelium plays a fundamental role in acute lung injury, particularly in the activation of pro-inflammatory cytokines. Some authors have proposed that the presence of interleukin-1 (IL-1) and TNF supports the presence of activated macrophages, which stimulate the production of other cytokines and increase the expression of adhesion molecules such as ICAM-1[39, 46, 47].

Little *et al*. showed that plasmatic levels of ICAM-1 in premature neonates at 14 days may be correlate with chronic neonatal lung disease and its severity[46]. Ballabh *et al*. reported similar findings of elevated ICAM-1 levels in neonates who developed CLD on approximately the 28th day of life[47]. In our study, the lung samples of premature neonates from the "new" CLD group had higher levels of ICAM-1 than the lung samples from the other groups. It reasonable to hypothesize that ICAM-1 is an early biomarker for CLD because it appears before the 28th day of oxygen supplementation, i.e., before a clinical diagnosis of CLD. These results contrast with the findings of other authors regarding the plasma levels of ICAM-1[39].

In our study, high levels of ICAM-1 in "new" CLD may have been the determining factor for the increased number of CD14+ cells in the alveolar septum. Wether this conclusion is correct, it is reasonable to assume that the increased number of CD14+ cells can influence the increase in apoptotic cascade activity, which seems to be a major cause of the reduced alveolar formation that occurs in "new" CLD. Further immunohistochemical and molecular studies are required to clarify this hypothesis[46, 47].

Few studies have correlated B-lymphocytes and VCAM with CLD, and no statistically significant differences in the number of CD20+ cells and VCAM immunoexpression were found between the groups in our study[44].

Our findings seems to corroborate the hypothesis that the initial inflammatory response is mediated by neutrophils and sustained by CD45RO+ and CD4+ T lymphocytes in the "classic" CLD form. Furthermore, the results suggest that CD14 may be a biomarker to connect inflammatory and apoptosis process, particularly in the lung injury associated with decreased alveolar volume observed in "new" CLD. Further studies are needed to better elucidate the pathogenesis of CLD, especially regarding the apoptotic process of the "new" CLD.

ETHICAL CONDUCT OF RESEARCH

The authors state that they have obtained approval of appropriate institutional review board and have followed the principles established by the Declaration of Helsinki for all human or animal experimental investigations. The research ethics board of the HC-UFPR reviewed and approved the study (registration number 1099.138/2005-08; approved on August, 30 2005).

RESUMO

Introdução: *A doença pulmonar crônica (DPC), conhecida clinicamente como displasia broncopulmonar, é uma das maiores causas de morbidade em neonatos que nasceram prematuros e foram submetidos à oxigenioterapia.* ***Objetivo:*** *Identificar moléculas inflamatórias em tecido pulmonar de recém-nascidos prematuros que morreram com DPC por meio do método de imuno--histoquímica.* ***Métodos:*** *Análise imuno-histoquímica de 51 amostras de pulmões de recém-nascidos prematuros – formando os grupos: sem DPC, DPC "nova" e DPC "clássica".* ***Resultados:*** *O influxo de neutrófilos e o número de células CD4+ e CD45RO+ foram maiores no grupo DPC "clássica" (*$p < 0,001$*).* ***Conclusão:*** *Os resultados sugerem que o processo inflamatório é mediado por neutrófilos e linfócitos CD45RO+ e CD4+ na DPC "clássica".*

Unitermos: *displasia broncopulmonar; imuno-histoquímica; nascimento prematuro; oxigenoterapia; neonatologia.*

REFERENCES

1. Northway WH, Rosan RC, Porter DY. Pulmonary disease following respiratory therapy of hyaline membrane disease. Bronchopulmonary dysplasia. N Engl J Med. 1967; 276: 357-68.

2. Bancalari E, Claure N. Definitions and diagnostic criteria for bronchopulmonary dysplasia. Semin Perinatol. 2006; 30: 164-70.

3. Rebello CM, Mascaretti RS. A "nova" displasia broncopulmonar. In: Manual da atualização em Neonatologia. 1st ed. Rio de Janeiro, RJ: Panamericana; 2004.

4. Sankar MJ, Agarwal R, Deorari AK, et al. Chronic lung disease in newborns. J Pediatr. 2008; 75(4): 369-76.

5. Bancalari E. Bronchopulmonary dysplasia: old problem, new presentation. J Pediatr. 2006; 82(1): 2-3.

6. Jobe AH. The new bronchopulmonary dysplasia. Curr Opin Pediatr. 2011; 23(2): 167-72.

7. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001; 163: 1723-9.

8. Ehrenkranz RA, Walsh MC, Vohr BR, et al. Validação da definição de displasia broncopulmonar do consenso do Instituto Nacional de Saúde. Pediatrics. 2006; 10(6): 413-22.

9. Walsh MC, Szefler S, Davis J, et al. Summary proceedings from the bronchopulmonary dysplasia group. Pediatrics. 2006; 117: 52-5.

10. Okamoto CT, Bahr JA, Silva LLG, et al. Análises histopatológica e morfométrica no diagnóstico da "nova" displasia broncopulmonar e comparação clinicopatológica com a forma clássica da doença. J Bras Patol Med Lab. 2009; 45(2): 147-52.

11. Tapia JL, Agost D, Alegria A, et al. Bronchopulmonary dysplasia: incidence, risk factors and resource utilization in a population of South American very low birth weight infants. J Pediatr. 2006; 82: 15-20.

12. Coalson JJ, Winter V, de Lemos RA. Decreased alveolarization in baboon survivors with bronchopulmonary dysplasia. Am J Respir Crit Care Med. 1995; 152: 640-6.

13. Coalson JJ, Winter VT, Siler-Khodr T, et al. Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med. 1999; 160: 1333-46.

14. Mascaretti RS, Mataloun MM, Dolhnikoff M, et al. Lung morphometry, collagen and elastin content: changes after hyperoxic exposure in preterm rabbits. Clinics. 2009; 64(11): 1099-04.

15. Bose CL, Dammann CEL, Laughon MM. Bronchopulmonary dysplasia and inflammatory biomarkers in the premature neonate. Arch Dis Child Fetal Neonatal. 2008; 93: 455-61.

16. Bourbon J, Boucherat O, Chailley-Heu B, et al. Control mechanisms of lung alveolar development and their disorders in bronchopulmonary dysplasia. Pediatr Res. 2005; 57: 38-46.

17. Santos CC, Slutsky A. The contribution of biophysical lung injury to the development of biotrauma. Annu Rev Physiol. 2006; 68: 585-618.

18. Santos CC, Zhang H, Liu M, et al. Bench-to-bedside review: biotrauma and modulation of the innate immune response. Critical Care. 2005; 9(3): 280-6.

19. Speer CP. Inflammation and bronchopulmonary dysplasia. Semin Neonatol. 2003; 8: 29-38.

20. Urlichs F, Speer CP. Neutrophil function in preterm and term infants. Neo Rev. 2004; 5: 417-29.

21. Ballabh P, Simm M, Kumari J, et al. Lymphocyte subpopulations in bronchopulmonary dysplasia. Am J Perinatol. 2003; 20(8): 465-75.

22. Willet KE, Jobe AH, Ikegami M, et al. Antenatal endotoxin and glucocorticoid effects on lung morphometry in preterm lambs. Pediatr Res. 2000; 48: 782-8.

23. Baurakiades E, Martins APC, Moreschi VN, et al. Histomorphometric and immunohistochemical analysis of infectious agents, T-cell subpopulations and inflammatory adhesion molecules in placentas from HIV-seropositive pregnant women. Diagn Pathol. 2011; 6: 101-8.

24. Costa Jr VHS, Baurakíades E, Azevedo MLV, et al. Immunohistochemistry analysis of pulmonary infiltrates in necropsy samples of children with non-pandemic lethal respiratory infections (RSV; ADV; PIV1; PIV2; PIV3; FLUa; FLUB). J Clin Virol. 2014; 61: 211-5.

25. Ambalavanan N, Carlo WA, D'Angio CT, et al. Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants. Pediatrics. 2009; 123: 1132-41.

26. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med. 2006; 11(5): 354-62.

27. Turato G, Zuin R, Saetta M. Pathogenesis and pathology of COPD. Respiration. 2001; 68: 117-28.

28. Gaga M, Bentley AM, Humbert M, et al. Increases in CD4+ T lymphocytes, macrophages, neutrophils and interleukin 8 positive cells in the airways of patients with bronchiectasis. Thorax. 1998; 53: 685-91.

29. Bochner BS. Road signs guiding leukocytes along the inflammation superhighway. J Allergy Clin Immunol. 2000; 106(5): 817-28.

30. O'Byrne PM. Airway inflammation and airway hyperresponsiveness. Chest. 1986; 90: 575-7.

31. Lindén A, Laan M, AndersonGP. Neutrophils, interleukin-17A and lung disease. Eur Respir J. 2005; 25: 159-72.

32. Lindén A. Interleukin-17 and airway remodeling. Pulm Pharmacol Ther. 2006; 19: 47-50.

33. Schmidt-Weber C, Akdis M, Akdis CA. Th17 cells in the big picture of immunology. J Allergy Clin Immunol. 2007; 120: 247-54.

34. Laan M, Linén A. IL-17 as a potential target for modulating airway neutrophilia. Curr Pharm Des. 2002; 8(20): 1855-61.

35. Laan M, Prause O, Miyamoto M, et al. A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha. Eur Respir J. 2003; 21(3): 387-93.

36. Ballard HO, Bernard P, Qualls J, et al. Azithromycin protects against hyperoxic lung injury in neonatal rats. J Invest Med. 2007; 55(6): 299-05.

37. Aghai ZH, Kode A, Saslow JG, et al. Azithromycin suppresses activation of nuclear factor-kappa B and synthesis of pro-inflammatory cytokines in tracheal aspirate cells from premature infants. Pediatr Res. 2007; 62(4): 483-8.

38. Ballard HO, Anstead M, Shook LA. Azithromycin in the extremely low birth weight infant for the prevention of bronchopulmonary dysplasia: a pilot study. Respir Res. 2007; 8: 41.

39. Bessler H, Komlos L, Punsky I, et al. CD14 receptor expression and lipopolysaccharide-induced cytokine production in preterm and term neonates. Biol Neonate. 2001; 80: 186-92.

40. Pedreira PR, Garcia-Prieto E, Albaiceta GM, et al. Respuesta inflamatória y apoptosis em La lesion pulmonar aguda. Med Intensiva. 2006; 30: 268-75.

41. Levy O. Innate immunity of the human newborn: distinct cytokine responses to LPS and other toll-like receptor agonists. J Endotoxin Res. 2005; 11(2): 113-6.

42. Henneke P, Osmers I Bauer K, et al. Impaired CD14-dependent and independent response of polymorphonuclear leukocytes in preterm infants. J Perinat Med. 2003; 31(2): 176-83.

43. Forster-Waldi E, Sadeghi K, Tamandl D, et al. Monocyte toll-like receptor 4 expression and LPS induced cytokine production increase during gestational aging. Pediatr Res. 2005; 58: 121-4.

44. Marsh LM, Cakarova L, Kwapiszewska G, et al. Surface expression of CD74 by type II alveolar epithelial cells: a potential mechanisms for macrophage migration inhibitory factor-induced epithelial repair. Am J Physiol Lung Cell Mol Physiol. 2009; 296(3): 442-52.

45. Kevill KA, Bhandari V, Kettunen M. A role for macrophage migration inhibitory factor in the neonatal respiratory distress syndrome. J Immunol. 2008; 180: 601-8.

46. Little S, Dean T, Bevin S, et al. Role of elevated plasma soluble ICAM-1 and bronchial lavage fluid IL-8 levels as markers of chronic lung disease in premature infants. Thorax. 1995; 50: 1073-9.

47. Ballabh P, Kumari J, Krauss AN, et al. Soluble E-selectin, soluble L-selectin and soluble ICAM-1 in bronchopulmonary dysplasia, and changes with dexamethasone. Pediatrics. 2003; 111(3): 461-8.

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4.3 ARTIGO 3

ANALYSIS OF INTERLEUKINS 6, 8, 10 AND 17 IN THE LUNGS OF PREMATURE NEONATES WITH BRONCHOPULMONARY DYSPLASIA.

Title Page

Analysis of interleukins 6, 8, 10 and 17 in the lungs of premature neonates with bronchopulmonary dysplasia

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Key words: bronchopulmonary dysplasia, interleukin, neonate, lung, biomarkers

Interleukins and bronchopulmonary dysplasia

Abstract

Bronchopulmonary dysplasia (BPD) is an abnormality in premature neonate lung development. The pathophysiology is uncertain and the inflammatory response to lung injury may be the pathway. **Objective:** To evaluate the role of interleukins 6, 8, 10 and 17 in BPD through anatomopathological and immunohistochemical study of the lungs of premature neonates with BPD. **Method:** Thirty two cases of neonatal necropsies from the Pathology Department of the Clinics Hospital of the Universidade Federal do Paraná performed between 1991 and 2005 were selected. The sample included neonates of less than 34 weeks of gestational age who underwent oxygen therapy and had pulmonary samples fixed in formalin and preserved in paraffin. Pulmonary specimens were later classified into 3 groups according to histopathological and morphometric changes (classic BPD, new BPD, and without BPD) and subjected to immunohistochemical analysis. The antibodies selected for the study were anti-IL-6, IL-8, IL-10 and IL-17 monoclonal antibodies. **Results:** IL-6, IL-8 and IL-10 showed no significant differences in tissue immunoexpression between the groups. IL-17 had greater tissue immunoreactivity in the group without BPD compared to the classic BPD group (1686 vs 866 μm^2, p = 0.029). **Conclusion** This study with lungs tissue of neonates with BPD showed that interleukins 6, 8 10 and 17 may not be involved in the pathophysiology of BPD. We speculated that IL-17 could be a protective factor for this disease.

Introduction

Worldwide, 8-10% of pregnancies progress to premature births. Approximately 25% of premature infants weighing less than 1500g develop bronchopulmonary dysplasia (BPD), impacting the morbidity and mortality of this group of patients[1,2].

This disease is the most common chronic lung lesion in preterm neonates. Those affected are who receive supplemental oxygen for at least 28 days[3,4], this is the clinical definition of BPD. The manifestations can range from minimal pulmonary changes to severe respiratory failure[5].

The anatomopathological presentation and definiton of BPD has two forms: the classic (old) form and the new form. For this pathological diagnosis, there is no need to wait 28 days as the clinical definition, the histopathological changes reveals the classification already at the time of the biopsy. The classic form is characterized by heterogeneity with severe airway epithelial lesions such as squamous metaplasia, marked airway smooth muscle hyperplasia, extensive alveolar septal fibrosis, and hypertensive remodeling of the pulmonary arteries[6,7,8]. This form is currently uncommon due to the application of novel therapeutic techniques such as less aggressive mechanical ventilation, antenatal corticosteroid use, surfactant administration and changes in delivery care[1,9,10]. However, classic BPD can still be observed in patients who require ventilation with high levels of oxygen and pressure[11]. All of the care practices that have been adopted in the management of prematurity have reduced the incidence of classic BPD, leading to a new form of BPD characterized by less heterogeneity, reduced and dysmorphic pulmonar vasculature with rare epithelial lesions and mild airway smooth muscle thickening. Another aspects

including a lower degree of airway injury, low lung inflammation and fibrosis, and large simplified alveolar structure with a reduced number of alveoli (hypoalveologenesis) are also observed in the new form of BPD[6,9,11,12,1,14] . An immature lung exposed to oxygen and mechanical ventilation lead to the release of inflammatory mediators, which damage the lungs by increasing pulmonary vascular permeability and attracting pro-inflammatory cytokines, increase levels of alveolar macrophages and neutrophils[5,15,16]. When this process occurs in the critical period of alveologenesis, the lung does not fully recover its growth potential, resulting in hypoalveologenesis and hypoplasia of the pulmonary vasculature[17]. Therefore, we selected proinflammatory interleukins 6, 8 and 17 and anti-inflammatory interleukin 10 for analysis. The aim of this study is to evaluate the role of interleukins in BPD through anatomopathological analysis of the lungs of premature neonates with classic and new BPD.

Methods

A total of 800 necropsy reports of neonates from the Pathology Department of the Clinics Hospital of the Universidade Federal do Paraná (Federal University of Paraná) from 1994 to 2005 were reviewed. The sample included 119 premature neonates, less than 34 weeks of gestational age, who had undergone oxygen therapy. Premature neonates with congenital malformations, chronic intrauterine diseases, and meconium aspiration syndrome were excluded. The study was approved by the ethics committee of the Clinics Hospital of the Universidade Federal do Paraná, under number 1099.138/2005-08, on August 30, 2005.

All the medical records of the selected premature neonates were analyzed, and all data related to the prenatal period and disease conditions were collected, including premature rupture of membranes (ROM), chorioamnionitis (clinical and/or confirmed by pathological examination of the placenta), gestational diabetes, hypertensive disease of pregnancy, Parkin score, Apgar scores at 1 and 5 minutes after birth, duration of mechanical ventilation and oxygen therapy, the presence of neonatal sepsis (clinical and/or laboratory testing with confirmed pathogen detection), necrotizing enterocolitis, use of postnatal antibiotics, patent ductus arteriosus (clinical and echocardiographic data), pneumonia, perinatal asphyxia, pulmonary hemorrhage, and pulmonary hypertension.

Of the 119 selected cases, 87 were excluded from the study (20 cases were excluded due to incomplete medical records, and 67 cases had inadequate necropsy material for immunohistochemical evaluation). Therefore, a total of 32 cases were included in the final sample.

Histologycal analysis

Five formalin fixed-paraffin embedded (FFPE) samples of each lung were collected (peripheral and pre-hilar regions of both lungs and all lobes), reexamined and classified into two groups, according to the histopathological changes (without taking into account clinical data): (A) cases with pathological features of classic BPD, characterized by varying degrees of pulmonary fibrosis, often alternating with over distended acini and major bronchial epithelial damage- 11 cases; (B) those who did not meet the criteria for classic BPD – 21 cases.

Morphometric analysis

Olympus BX 50 microscope (Olympus Optical Co. LTDA, Japan) linked to a Dinoeye camera and a computer were used to obtain 10 medium power field photomicrographs (200x) per case (the total area of medium power field is 475.439 square micrometers). These photomicrographs (200x) were subjected to the morphometric analysis, using Image Pro Plus software. All alveoli in each medium power field were counted and their perimeters were measured (micrometers). Mean values (number of alveoli and perimeter) for each patient were used for statistical analysis (TABLE 1).

This analysis was performed by a single observer without prior knowledge of the oxygen therapy data. The observed patterns were compared with the normal parameters found in the literature[18.19] and with the criteria for diagnosis of the new form of BPD, and the cases that fit the description of BPD were distinguished from normal cases. The cases that were considered normal (i.e., without BPD) represent the "control group" of this study. Therefore, 3 groups were formed: (1) classic BPD, n = 11; (2) new BPD, n = 5; and (3) without BPD, n = 16[20,21].

Immunohistochemical analysis

Tissue microarrays were assembled from lung samples from all the cases and analyzed immunohistochemically (peripheral and pre-hilar regions, 4 samples for each case with 3mm diameter each one), and the samples were evaluated by immunohistochemistry[20-22].

All samples were stained in duplicate, with one slide serving as a negative control and one serving as a positive control. Methyl alcohol and hydrogen peroxide were used to block endogenous peroxidase initially, and then distilled water plus hydrogen peroxide were used for the second blockade.

The primary antibodies used in this study were: anti-IL-6 is a mouse monoclonal antibody, clone not available, dilution 1:200, Abcam^{TM}, Cambridge, United Kingdon; anti-IL-8 is a mouse monoclonal antibody, clone not available, dilution 1:200, Abcam^{TM}, Cambridge, United Kingdon; anti-IL-10 is a mouse monoclonal antibody, clone JES3-12G8, dilution 1:200, ABR^{TM}, Goden, United States of America; anti-IL-17 A is a rabbit monoclonal antibody, clonenot available, Abcam^{TM}, Cambridge, United Kingdon. These antibodies were incubated overnight and staining was performed using dextran polymer-conjugated secondary antibody for 30 minutes. For staining, the DAB + substrate complex was added to the slides, and counter-staining was performed with Mayer's hematoxylin. Canadian Balsam was used to mount the slides. Negative controls (without primary antibody) and positive controls were used for each reaction.

The immunostained slides were analyzed morphometrically with the Olympus BX50 optical microscope and Image Pro Plus® software, with evaluation of the immunostained area and the staining intensity. Positivity, represented by brown coloring, was converted into the immunopositive area (square micrometers - μm^2). For this analysis, a minimum of 30 and a maximum of 50 photomicrographs at 400x magnification were taken for each case, and the results were expressed in area (μm^2) per high-magnification field. We excluded the photomicrographs that had artifacts, poor quality, bronchial cells

and with did not have enough material. The alveolar immunopositive cells were evaluated (type II pneumocyte and intracellular macrophage).

Statistical analysis

Quantitative variables were described as the means, medians, minimum values, maximum values and standard deviations. Qualitative variables were described as frequencies and percentages. The non-parametric Kruskal-Wallis test was used to compare the groups classified by bronchopulmonary dysplasia (classic BPD, new BPD and without BPD) in terms of the quantitative variables. For the qualitative variables, the groups were compared pairwise using Fisher's exact test. $p < 0.05$ indicated statistical significance. In the multiple comparisons performed using Fisher's exact test, the level of significance was Bonferroni-corrected $p < 0.017$. The data were analyzed using IBM SPSS Statistics v. 20 software.

Results

The cases of classic BPD (n= 11) presented the following histopathological features: (a) hyaline membranes, bronchial necrosis, bronchiolitis obliterans, bronchiectasis, and early septal fibrosis were observed in the acute phase; and (b) prominent interstitial fibrosis, thickening of the vascular wall, and collapsed and hyperdistended acini were observed in the chronic phase.

The remaining 21 cases (new BPD, n= 5; without BPD, n= 16) presented minimal histopathological changes, such as mild septum edema, altered alveolar insufflation. From a histopathological perspective (without clinical data),

the group with new BPD could not be distinguished from the group without BPD; therefore, a morphometric analysis of the lungs was required (TABLE 1). The group with new BPD presented a smaller number of alveoli compared to the group without BPD. The oxygen exposure time of the premature lungs and the survival time of the cases in each group were exposed and described in TABLE 2. The neonates in the new BPD group were heavier (a mean weight of 1340g) and more mature (a Parkin score of 33.4 weeks) relative to those in the other 2 groups, especially compared to those in the group without BPD (a mean weight of 957.8g and a Parkin score of 28.8 weeks). The classic BPD group had the worst Apgar scores. Neither gender predominated in the 3 groups (TABLE 3).

TABLE 4 show prenatal data, including ROM (greater than 18 hours), clinical chorioamnionitis, gestational diabetes, and hypertensive disease of pregnancy, as well as postnatal data, including the presence of pneumonia, pulmonary hemorrhage, pulmonary hypertension, perinatal asphyxia, neonatal sepsis, necrotizing enterocolitis, patent ductus arteriosus and antibiotic use.

The immunohistochemical analysis of ILs 6, 8, 10 and 17 is presented in TABLE 5 and FIGURE 1 and 2.

Discussion

BPD has a multifactorial etiology, but the persistent inflammatory process may be the factor that most contributes to the development of chronic pulmonary diseases[23]. The inflammatory response in a developing lung causes vascular, alveolar and bronchial growth changes[24-26]. So in this study, we investigated the role of some ILs in the pathophysiology of BPD trough immunohistochemical analysis of lung tissue samples (necropsy) of neonates

who had received treatment with oxygen and mechanical ventilation. This neonates survival a short time (TABLE 2), but enough time for happens the alveolar cells damage, seen in anatomopathological study. Some studies demonstrated that elevated levels of several inflammatory cytokines during the first week of premature neonate life were associated with development of BPD[27-29]. The level of IL-6 and IL-8 in the tracheal aspiration and serum in the first day of life, were persistently increased on the 28th day of life in very low birth weight with BPD[30]. The group without BPD was more immature (TABLE 3) and which had less exposure to oxygen due to early death, maybe not having time to develop the characteristic lesions of BPD, this group was the control.

The collection site of interleukins is an important factor for analysis. The cytokine profiles in cord blood, amniotic fluid and tracheal aspirate may not reflect the actual situation in the lungs; therefore, evaluation in human lung tissue is essential to better represent the circumstances of the disease [31]. Related studies with lung tissue samples are virtually non-existent in the literature. Studying the inflammatory response in necropsy material is advantageous because the material is controlled and allows an unbiased analysis of the data, but this material is disadvantageous due to the preparation of the material, which is FFPE, complicating the search for secreted and fragile molecules such as interleukins and interfering in genetic studies[32-34].

Factors antenatal, natal, or postnatal can interrupt pulmonary vascular and alveolar development; trigger an inflammatory response and cell injury contributing to BPD[2,6,35]. Because this, we evaluated some antenatal, natal, and postnatal factors. Some antenatal factors such as premature ROM, clinical chorioamnionitis and/or anatomopathological changes of the placenta, which

may be correlated with the onset of BPD according to the international literature[6,35,36,37]. However, the data in this study did not show increase of incidence of BPD associated with this factors (TABLE 4). The specific hypertensive disease of pregnancy is not a risk factor to BPD, confirm in this study and previous study[6].

Regarding the postnatal factors that may influence the onset of BPD, some studies suggest that postnatal infection is a more important predictor of BPD than antenatal inflammation[2,6]. The classic BPD group included a higher number of premature infants with pneumonia, sepsis, necrotizing enterocolitis and patent ductus arteriosus showing the presence of systemic inflammatory response and cell injury in this group (TABLE 4).

The BPD is the result from an imbalance between lung injury and repair in the developing lung, so an imbalance between "proinflammatory" and "anti-inflammatory" factors is believed to exist, which involves inflammatory cells, cytokines and an arsenal of humoral mediators in the airways and lung tissues of the premature infant.[6,35,37]. The increase of IL-6 in blood cord was associated with greater risk of development sepsis, pneumonia, necrotizing enterocolitis, and BPD. This systemic inflammatory response was described as Fetal inflammatory response syndrome (FIRS)[38,39].

IL-6 is a proinflammatory cytokine mediating acute lung injury and exacerbating ventilator-induced lung injury[30,40]. IL-6 is also involved in hematopoiesis, regulation of bone absorption, growth cells, differentiation survival, apoptosis and cell proliferation. Is produced by some cells like monocytes, fibroblasts and endothelial cells[41-43]. The IL-6 receptor is widely distributed in fetal tissue, including bronchial epithelial cells[43].

The IL-6 plays an important role in lung inflammation in preterm newborns and serves as a risk factor for the development of BPD when found in high concentrations in tracheal aspirate and/or cord blood [30,44]. However, conflicting results have been found in studies of IL-6. One study demonstrated a cellular protective effect of IL-6 against exposure to oxygen in adult lung tissues of mice; this result may have been related to the use of mature lungs[45]. Another study showed that elevation of IL-6 and/or IL-8 promotes lung maturation by synthesis of surfactant protein A[46]. Fetal IL-6 is a cytokine that regulates lung surfactant proteins, which are very important in lung maturation. It also regulates angiogenesis and morphogenesis of the lung[47,48], decreasing the incidence of respiratory distress syndrome in newborns[49]. In our study, the immunoexpression of IL-6 had a mean the 7056 in the group without BPD, compared with 5624 in the new BPD group (p 0,183), not showing the presence of IL-6 in BPD group (TABLE 5).

IL-8 is a potent chemotactic agent capable of recruiting neutrophils, and the presence of neutrophils in tracheal aspirate is associated with chronic lung diseases[23]. It is a member of the CXC family and is synthesized by a variety of cells: alveolar macrophages, endothelial cells, epithelial cells, fibroblasts etc[50]. The elevated IL-8 in cord blood is associated with death and moderate and severe BPD, and its increased expression in bronchoalveolar aspirate could be associated with abnormal lung development and maturation in neonates with BPD[50,51,52]. The high levels of IL-8 in tracheal aspirate were correlated with increased duration of ventilation in small preterm infants[53]. In our study, when we compare the group with BPD (1584) and without BPD (1790),

no statistical significance was observed. Inflammatory process wasn't observed in any group (TABLE 5).

IL-10 is a potent anti-inflammatory cytokine, and has a inhibitory effect on inflammation[54-56]. It is produced by macrophages and T-helper type II lymphocytes, which control the production of tumor mediators through the stimulation of epithelial cells of the immune system[56]. They are responsible for the production, differentiation and proliferation of B cells and macrophages[31].

Reduced levels of IL-10 in the placenta and tracheal aspirate are found in neonates who develop BPD[57]. IL-10 improve lung tissue oxygenation and inhibits oxidative stress, reducing ventilator-induced lung injury and inflammation[58,59]. In 2011, Hawwa showed that mechanical stretch of lung structures caused by mechanical ventilation and the release of IL-1β, IL-6, MCP-1, RANTES and TNF-α and IL-10 administration before stretch occurred inhibited their release, indicating that IL-10 protected the lungs of premature infants under mechanical ventilation[54,55]. Also in 2011, Lee showed a decrease in IL-10 production in the presence of hyperoxia, and prior cell incubation with recombinant IL-10 protected against the damage caused by oxygen[60]. Maybe, IL-10 is a protective factor against the development of BPD[54,55,57,60]. However, some premature infants are not able to activate anti-inflammatory cytokines, such as IL-10, so they are more predisposed to a more pronounced inflammatory response[53].

In a studied, the use of IL-10 as an inhibitor of the inflammatory cascade in BPD, this interleukin proved to be more potent to block IL-8 than the glucocorticoids used in the current therapy of the disease[61]. In contrast, one study with extreme preterms have shown that IL-10 expression in the

pulmonary aspirate is increased soon after birth and is related to the incidence of BPD and its increased expression along with the presence of chorioamnionitis also raises the incidence of the disease[62]; and another study showed high serum IL-10 and IL-8 dosage was related to BPD[63] associated with death[29]. A genetic study with IL-6 and IL-10 showed no relationship between these interleukins and BPD[64]. Our study, with human lung tissue samples, showed similar results (TABLE 5). Therefore, more studies are needed to clarify the true role of IL-10 in BPD.

IL-17 is a known proinflammatory cytokine[65] that regulates neutrophil production and tissue inflammatory reactions[29], is involved in pulmonary inflammatory processes, and is an important mediator of endothelial permeability[66-68]. Assists in the defense of the immune system against extracellular pathogens and is produced by the T lymphocyte[65] (Th17) and by innate immune cells. These cells are strategically located in barrier tissues that protect the human body from the external environment and provide vital host protections against microorganisms (mainly bacteria and fungi)[69,70]. When exposed to a microorganism, these innate immune cells increase the production of IL-17A in 4 at 8 hours, attracting neutrophils to inflamed sites to accelerate the death and elimination of aggressive microorganisms[71]. IL-17 has been implicated in the development or pathogenesis of various autoimmune and inflammatory diseases[72].

Neonates have a reduced baseline IL-17A production compared to adults, which may decrease the neonatal immune response and contribute to their increased susceptibility to infection[73,74,75] by group B Streptococcus, Escherichia coli[69], Klebsiella pneumoniae[76] and Candida albicans[70]. The

classical BPD group had a higher incidence of pneumonia, necrotizing enterocolitis, and sepsis, perhaps because it had the lowest IL-17 immunoexpression.

IL-17A levels are similar between preterm and term infants, their production do not depend on immunological maturation or of gestational age[73]. Production of IL17A in the embryonic thymus begins around 24-28 weeks of gestation. IL-17 is important for the immunological surveillance and protection of the neonate, there is evidence that IL-17 plays a prominent role in neonatal pathology[75].

Mikacenic et al. in 2016, demonstrated that circulating and alveolar IL-17 was elevated in humans with distress respiratory syndrome (ARDS), but the role of the Th-17 immune response in ARDS has not yet been defined[77]. Studies with anti-IL-17 have shown some efficacy in the protection of lungs from the inflammatory effects to lipopolysaccharide-induced acute lung injury[78,79]. In our study, IL-17 had an important expression in the without BPD group compared to the classical BPD group (1686 vs 866 μm^2, $p = 0.029$), suggesting a protective response (TABLE 5).

Our sample also did not have significant chorioaminionitis (1 case in the classical group, and 2 cases in the group without BPD), thus not influencing our result (TABLE 4). Some studies suggest the association of chorioamnionites with an increased IL-17 and systemic inflammatory responses[25,26,80,81]. This inflammatory state probably involved in neonates diseases such as sepsis, necrotizing enterocolitis, retinopathy of prematurity[35,37,75], in BPD was not confirmed. Some authors suggests the fetal inflammatory response can be protective for chronic lung disease[82]. Sood and col. in 2012, analyzed the

presence of IL-17 in sepsis and didn't find difference between the groups with and without infection[74]. Ambalavanan in 2009 found a similar result as ours, the presence of a higher concentration in the blood of neonates at day 0, 3, 7, 14 of IL17 was associated with survival without BPD, and they speculate that a reduction in IL-17 may possibly inhibit angiogenesis and thereby attenuate alveolar development[29].

The true role of ILs in BPD is not yet known. In our study IL-17 showed a trend as a protective factor against the development of BPD. It is known that IL-17A contributes to the maintenance of a healthy respiratory epithelial border, promoting the production of adhesion molecules and cellular junction through the induction of ICAM-1 by airway epithelial cells[83,84,85]. Therefore, strict regulation of IL-17A seems vital for proper maintenance of the border of the respiratory epithelium, while altered production may impair lung tissue development, resulting in harmful pulmonary pathologies in neonates[75], and maybe because of this function of IL-17A we observed this result. The regulation of IL17 is fundamental for the homeostasis of the individual; therefore further studies should be performed. Its increase could be deleterious (persistent inflammatory processes) or its absence could be fatal (difficulty in combating extracellular microorganisms)? Prematurity is still a challenge for science, the role of IL in neonatal diseases needs further studies, in search for biomarkers and therapeutic tools.

This study has limitations such as the short life time and immaturity of the group without BPD, and use of neonates tissue from necropsy. These neonates may have had other insults involved in the death that were not seen in the analysis of pre-natal and postnatal data.

Conclusion

This study with neonate lung tissue with BPD showed that interleukins 6, 8, 10 and 17 may not be involved in the pathophysiology of BPD. IL-17 showed greater expression in the group without BPD, we speculated that it could be a protective factor. More studies are needed to assess ILs role as a biomarkers and even as a therapeutic factors for BPD.

References

1. O´Reilly M, Sozo F, Harding R. The impact of preterm birth and bronchopulmonary dysplasia on the developing lung: long- term consequences for respiratory health. *Clin exp pharmacol physiol*. 2013; 16: 2.

2. Jensen E A, Schmidt B. Epidemiology of bronchopulmonary dysplasia. *Birth defects Res a Clin Mol Teratol*. 2014; 100: 145-157.

3. Jobe AH, Bacalari E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med*. 2001; 163: 1723 -1729.

4. Gomez Pomar E, Concina VA, Samide A, Westgate PM, Bada HS. Bronchopulmonary Dysplasia: Comparison Between the Two Most Used Diagnostic Criteria*. Z Front Pediatr.* 2018; 12; 6; 397.

5. Tannuri U, Freddi NA. Displasia broncopulmonar. In: Carvalho W B, Hirschheimer M R, Proença J O F, Freddi N A, Troster E J. Ventilação Pulmonar Mecânica em Pediatria e Neonatologia. São paulo: Editora Atheneu. 2004; 559-566.

6. Thekkeveedu RK, Guaman MC, Shivanna B. Bronchopulmonary dysplasia: A Review of Pathogenesis and Pathophysiology. *Respir Med*. 2017; 132; 170-177.

7. Northway WH Jr, Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N Engl J Med.* 1967; 16: 276: 357-68.

8. Bonikos DS, Bensch KG, Northway WH, Jr, Edwards DK. Bronchopulmonary dysplasia: the pulmonary pathologic sequel of necrotizing bronchiolitis and pulmonary fibrosis. *Human pathology*. 1976; 7: 6: 643–666.

9. Cerny l, Torda JS, Rehan VK. Prevention and treatment of bronchopulmonary dysplasia: contemporary status and future outlook. *Lung.* 2008; 186: 75-89.

10. Baraldi E, Fillipone M. Chronic lung disease after premature birth. *N Engl J Med*. 2007; 357: 1946-1955.

11. Monte LFV, Silva Filho LVF, et al. Displasia broncopulmonar. *J Pediatr*. 2005; 8: 99-110.

12. Bhandary A, Panitch HB. Pulmonary outcomes in bronchopulmonary dysplasia. *Seminperinatol*. 2006; 30: 219-226.

13. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. *Hum Pathol.* 1998; 29: 710-707.

14. Coalson JJ. Pathology of new bronchopulmonary dysplasia. *Semin Neonatol.* 2003; 8: 73-81.

15. Woods SJ, Waite AA, O'Dea KP, et al. Kinetic profiling of *in vivo* lung cellular inflammatory responses to mechanical ventilation. *Am J Physiol Lung Cell Mol Physiol.* 2015; 308: 912–921.

16. Tang X. Interleukin-33 (IL-33) Increases Hyperoxia-Induced Bronchopulmonary Dysplasia in Newborn Mice by Regulation of Inflammatory Mediators. *Med Sci Monit.* 2018; 24: 6717–6728.

17. Massaro D, Massaro GD. Critical period for alveologenesis and early determinants of adult pulmonary disease. *Am J Physiol Lung Cell Molphysiol*. 2004; 287: 715-717.

18. Coalson JJ, Winter V, Lemos RA. Deacrease alveolarization in baboon survivors with bronchopulmonary dysplasia. *Am J Resp Crit Care Med*. 1995; 152: 640-646.

19. Coalson JJ, Winter V, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. *Am J Resp Crit Care Med*. 1999; 160: 1333-1346.

20. Witkowski SM, Noronha L, Okamoto CT, et al. Immunohistochemical analysis of apoptosis and cell proliferation in lungs of premature infants with chronic lung disease (bronchopulmonary dysplasia). *J. Bras. Patol. Med. Lab.* 2016; 52: 407-415.

21. Okamoto CT, Oldenburg Neto CF, Witkowski SM, et al. Quantitative analysis of inflammatory and adhesion molecules in lungs of neonates with chronic lung disease (bronchopulmonary dysplasia) receiving mechanical ventilation. *J. Bras. Patol. Med. Lab.* 2016; 52: 253-261.

22. Kumar B, de Silva M, Venter DJ, Armes J E. Tissue microarrays: a practical guide. *Pathology*. 2004; 36: 295-300.

23. Su BH, Chiu HY, Lin TW, Lin HC. Interleukin-8 in bronchoalveolar lavage fluido f premature infants at risk of chronic lung disease. *J formos med assoc*. 2005; 104: 244-248.

24. Laughon MM, Smith PB, Bose C. Prevention of bronchopulmonary dysplasia. *Seminars in fetal and neonatal care*. 2009; 14: 374-382.

25. Kunzmann S, Collins JJ, Kuypers E, Kramer BW. Thrown off balance: effect of antenatal inflammation on the developing lung and immune system. *Am j obstetgynecol*. 2013; 1.

26. Choi CW, Beyond K, et al. Decrease expression of transforming growth factor-beta 1 in bronchoalveolar lavage cells of preterm infants with maternal chorioamnionitis. *J korean med sci*. 2008; 23: 609-615.

27. Zhang ZQ, Huang XM, Lu H. Early biomarkers as predictors for bronchopulmonary dysplasia in preterm infants: a systematic review. *Eur J Pediatr*. 2014; 173: 15-23.

28. Köksal N, Kayik B, Çetinkaya M. et al. Value of serum and bronchoalveolar fluid lavage pro- and anti-inflammatory cytokine levels for predicting bronchopulmonary dysplasia in premature infants. *Eur Cytokine Netw*. 2012; 23: 29–35.

29. Ambalavanan N, Carlo WA, D'angio CT, et al .Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants. *Pediatrics.* 2009; 123: 1132–1141.

30. Hsiao CC, Chang JC, Tsao IY, et al. Correlates of elevated interleukin-6 and 8-hidroxy-2-deoxyguanosine levels in tracheal aspirates from very low birth weight infants who develop bronchopulmonary dysplasia. *Pediatrics and Neonatology*. 2017; 58: 63-69.

31. Speer C P. Inflammation and bronchopulmonary dysplasia. *Semin neonatol*. 2003; 8: 29-38

32. Mcfarlane MJ, Feinstein AR, Wells CK, Chan CK. The epidemiologic necropsy: unexpected detections, demographic selections and changing rates of lung cancer. *Jama*. 1987; 258: 331-338.

33. Forghani B, Dennis J. Immunoenzymatic staining. In: Schimdt NJ, Emmons R W. Procedures for rickettsial and chlamydial infections. *American Public Health Association*. 1989; 135-156.

34. Simon R, Mirtacher M, Sauter G. Tissue microarrays. *Biotechniques*. 2004; 36: 98-105.

35. Speer CP. Pre-and postnatal inflammatory mechanisms in chronic lung disease of preterm infants. *Pediatrresp reviews*. 2004; 5: 241-244.

36. Metcalfe A, Lisonkova S, Sabr Y, Stritzke A, Joseph KS. Neonatal respiratory morbidity following exposure to chorioamnionitis. *BMC Pediatr*. 2017; 17: 128.

37. Ryan RM, Ahmeq Q, Lakshminrusimha S. Inflammatory mediators in the immunobiology of bronchopulmonary dysplasia. *Clin RevAallergy Immunol*. 2008; 34: 174-190.

38. Gomez R, Romero R, Ghezzi F, Yoon BH, Mazor M, Berry SM. The fetal inflamatory response syndrome. *Am J Obstet Gynecol*.1998; 179: 195-202.

39. Ozalkaya E, Karatekin G, Topcuoğlu S, Gürsoy T, Ovalı F. Morbidity in preterm infants with fetal inflammatory response syndrome. *Pediatr Int*. 2016; 58:850-854.

40. Hillman NH, Polglase GR, Pillow JJ, Saito M, Kallapur SG, Jobe AH. Inflammation and lung maturation from stretch injury in preterm fetal sheep. *Am J Physiol Lung Cell Mol Physiol.* 2011; 300: 232-241.

41. Heinrich PC, Behrmann I, Haan S, et al. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J*. 2003; 374: 1-20.

42. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M. IL-6 an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory response. *J Clin Invest*. 1998; 101: 311-320.

43. Dame JB, Juul SE. The distribution of receptors for the pro-inflammatory cytokines interleukin (IL-6 and IL-8) in the developing human fetus. *Early Hum Dev*. 2000; 58: 25-39.

44. Von Bismarck P, Class A, Schickor C, et al. Altered pulmonary interleukin-6 signaling in preterm infants developing bronchopulmonary dysplasia. *Exp Lung Res*. 2008; 34: 694-706.

45. Ward NS, Waxman AB, Homer RJ, et al. Interleukin-6 induced protection in hiperoxic acute lung injury. *Am Respir Cell Mol Bio*. 2000; 22: 535-542.

46. Ikegami T, Tsuda A, Karube A, Kodama H, Hirano H, Tanaka T. Effects of intrauterine IL-6 and IL-8 on the expression of surfactant apoprotein mRNAs in the fetal rat lung. *Eur J Obstet Gynecol Reprod Biol.* 2000; 93: 97-103.

47. Mcclintock JK, Wagner EM. Role of IL-6 in systemic angiogenesis of the lung. *J Applphysiol*. 2005; 99: 861-866.

48. Parera MC, et al. Distal angiogenesis: a new concept for lung vascular morphogenesis. *Am J Physiol Lung Cell Molphysiol*. 2005; 288: 141-149.

49. Shimoya K, Taniguchi T, Matsuzaki N, et al. Chorioamminionitis decrease incidence of respiratory distress syndrome by elevanting fetal interleukin-6 serum concentration. *Hum Reprod*. 2000; 15: 2234-2240.

50. Mukaida N. Pathophysiological roles of interleukin 8/cxcl8 in pulmonary diseases. *Am J Physiol Lung Cell Molphysiol*. 2003; 284: 566-577.

51. Liu DY, Wu J, Zhang XY, Feng ZC. Expression of IL-8, SP-a and tgf-beta1 in bronchoalveolar lavage fluid of neonates with bronchopulmonary dysplasia. *Zhongguo Dang Dai*. 2010; 12: 444-446.

52. Chakraborty M, McGreal EP, Williams A, Davies PL, Powell W, et al. Role of Serine Proteases in the Regulation of Interleukin-8_{77} during the Development of Bronchopulmonary Dysplasia in Preterm Ventilated Infants. *Plos one*. 2014; 9: 114524.

53. De Dooy J, Ieven M, Stevens W, De Clerck L, Mahieu L. High levels of CXCL8 in tracheal aspirate samples taken at birth are associated with

adverse respiratory outcome only in preterm infants younger than 28 weeks gestation. *Pediatr Pulmonol*. 2007; 42:193–203.

54. Hawwa RL, Hokenson MA, Wang Y, et al. Differential expression of mmp-2 and -9 and their inhibitor in fetal lung cells exposed to mechanical stretch: regulation by IL-10. *Lung*. 2011; 189: 341-349.

55. Hawwa RL, Hokenson M A, Wang Y, et al. IL-10 inhibits inflammatory cytokines releases by fetal mouse lung fibroblasts exposed to mechanical strech. *Pediatrpulmonol*. 2011; 46: 640-649.

56. Baier JR, Loggins J, Yanamandra K. IL-10, IL-6 and CD14 polymorphisms and sepsis outcome in ventilated very low birth weight infants. *BMC Medice*. 2006; 4: 10.

57. MCgowan EC, Kostadinov S, et al. Placental IL-10 dysregulation and association with bronchopulmonary dysplasia risk. *Pediatr res*. 2009; 66: 455-460.

58. Chen J, Lin J, Luo H, Li M. Effects of Human Interleukin-10 on Ventilator-Associated Lung Injury in Rats. Inflammation. 2018; doi: 10.1007/s10753-018-0911-7

59. Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev*. 2008; 226:205-218.

60. Lee HS, Kim CK. Effect of recombinant IL-10 on cultured fetal rat alveolar type ii cells exposed to 65%-hiperoxia. *Respiratory research*. 2011; 12: 68-83.

61. Davidson D, Patel H, Degoy AC, et al. Differential effect of exogenous interleukin-10 versus glucocorticoids on gene expression and pro-inflammatory cytokine relase by polymorphonuclear leukocytes and monocytes of the newly born. *Am J transl res*. 2013; 5: 103-115.

62. Hikino S, Ohga S, Kinjo T, et al. Tracheal aspirate gene expression in preterm newborn and development of bronchopulmonary dysplasia. *Pediatrint*. 2012; 54: 208-214.

63. Paananen R, Husa AK, Vuolteenaho R, et al. Blood cytokines during the perinatal period in very preterm infants: relationship of inflammatory response and bronchopulmonary dysplasia. *J Pediatr*. 2009; 154:1.

64. Huusko JM, Karjalainen MK, Mahlman M, et al. A study of genes encoding cytokines (IL6, IL10, TNF), cytokine receptors (IL6R, IL6ST), and glucocorticoid receptor (NR3C1) and susceptibility to bronchopulmonary dysplasia. *BMC Med Genet*. 2014;15:120.

65. Rajita P, Carrozzi VR, Sambandam A. The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases. *Immunology*. 2011; 134: 8-16.

66. You QH, Sun GY, Shen JL, Wang Y. Interleukin-17f-induced pulmonary microvascular endothelial monolayer hypermeability via the protein kinase c pathway. *Surg res*. 2010; 162: 110-121.

67. Fogli IK; Sundrud MS, Goel S, et al. T-cell-derived il-17 mediates epithelial changes in the airway and drives pulmonary neutrophilia. *J Immunol*. 2013; 191: 6.

68. Linden A, Laan M, Anderson G P. Neutrophils, interleukin-17a and lung disease. *Eur respir j.* 2005; 25: 159-172.

69. Caron JE, La Pine TR, Augustine NH, Martins TB, Kumánovics A, Hill HR. Severely depressed interleukin-17 production by human neonatal mononuclear cells. *Pediatr Res*. 2014; 76:522–527.

70. Huang W, Na L, Fidel PL, Schwarzenberger P. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J Infect Dis*. 2004;190: 624–631.

71. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol*. 2010;10 :479–489.

72. Sakaguchi R, Chikuma S, Shichita T, et al. Innate-like function of memory Th17 cells for enhancing endotoxin-induced acute lung inflammation through IL-22. *Int. Immunol.* 2016; 28: 233–243.

73. Schelonka RL, Maheshwari A, Carlo WA, et al. NICHD Neonatal Research Network T cell cytokines and the risk of blood stream infection in extremely low birth weight infants. Cytokine. 2011; 53: 249–55.

74. Sood BG, Shankaran S, Schelonka RL, et al. Cytokine profiles of preterm neonates with fungal and bacterial sepsis. *Pediatr Res*. 2012; 72: 212-220.

75. Lawrence SM, Ruoss JL, Wynn JL. IL-17 in Neonatal Health and Disease *Am J Reprod Immunol*. 2018; 79: e12800.

76. Happel KI, Zheng M, Young E, et al. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to Klebsiella pneumoniae infection. *J Immunol*. 2003; 170: 4432–4436.

77. Mikacenic C, Hansen EE, Radella F, Gharib SA, Stapleton RD, Wurfel MM. Interleukin-17A Is Associated With Alveolar Inflammation and Poor Outcomes in Acute Respiratory Distress Syndrome. *Crit Care Med*. 2016; 44: 496–502.

78. Righetti RF, Santos TM, Camargo IM, et al. Protective effects of anti-il17 on acute lung injury induced by lps in mice. *Front Pharmacol.* 2018; 9: 1021.

79. Li TJ, Zhao LL, Qiu J, et al. Interleukin-17 antagonist attenuates lung inflammation through inhibition of the erk1/2 and nf-κb pathway in lps-induced acute lung injury. *Mol Med Rep*. 2017; 16: 2225-2232.

80. Gleditsch DD, Shornick LP, Van Steenwinckel J, Gressens P, Weisert RP, Koenig JM. Maternal inflammation modulates infant immune response patterns to viral lung challenge in a murine model. *Pediatr Res*. 2014; 76: 33–40.

81. Rito DC, Viehl LT, Buchanan PM, Haridas S, Koenig JM. Augmented Th17-type immune responses in preterm neonates exposed to histologic chorioamnionitis. *Pediatr Res*. 2017;81:639-645.

82. Lahra M M, Beeby PJ, Jeffery HE. Intrauterine inflammation, neonatal sepsis, and chronic lung disease: a 13-year hospital cohort study. *Pediatrics*. 2009; 123:1314-1319.

83. Kawaguchi M, Kokubu F, Kuga H, et al. Modulation of bronchial epithelial cells by IL-17. *J Allergy Clin Immunol*. 2001; 108: 804–809.

84. Kinugasa T, Sakaguchi T, Gu X, Reinecker HC. Claudins regulate the intestinal barrier in response to immune mediators. *Gastroenterology*. 2000; 118:1001–1011.

85. Tsai HC, Velichko S, Hung LY, Wu R. IL-17A and Th17 cells in lung inflammation: an update on the role of Th17 cell differentiation and IL-17R signaling in host defense against infection. *Clin Dev Immunol*. 2013. doi: 10.1155/2013/267971

Figure 1. Immunohistochemical analysis of interleukins 6 and 8 (40x objective).

A-IL-6 classic BPD, **B**- IL-6 new BPD, **C**- IL-6 without BPD, **D**- IL-8 classic BPD, **E**- IL-8 new BPD, **F**- IL8 without BPD. Demonstrating immunopositive type I and II pneumocytes (arrows) and immunopositive alveolar macrophages (arrows heads).

Figure 2. Immunohistochemical analysis of interleukins 10 and 17 (40x objective).

A-IL-10 classic BPD, **B**- IL-10 new BPD, **C**- IL-10 without BPD, **D**- IL-17 classic BPD, **E**- IL-17 new BPD, **F**- IL17 without BPD. Demonstrating immunopositive type I and II pneumocytes (arrows) and immunopositive alveolar macrophages (arrows heads).

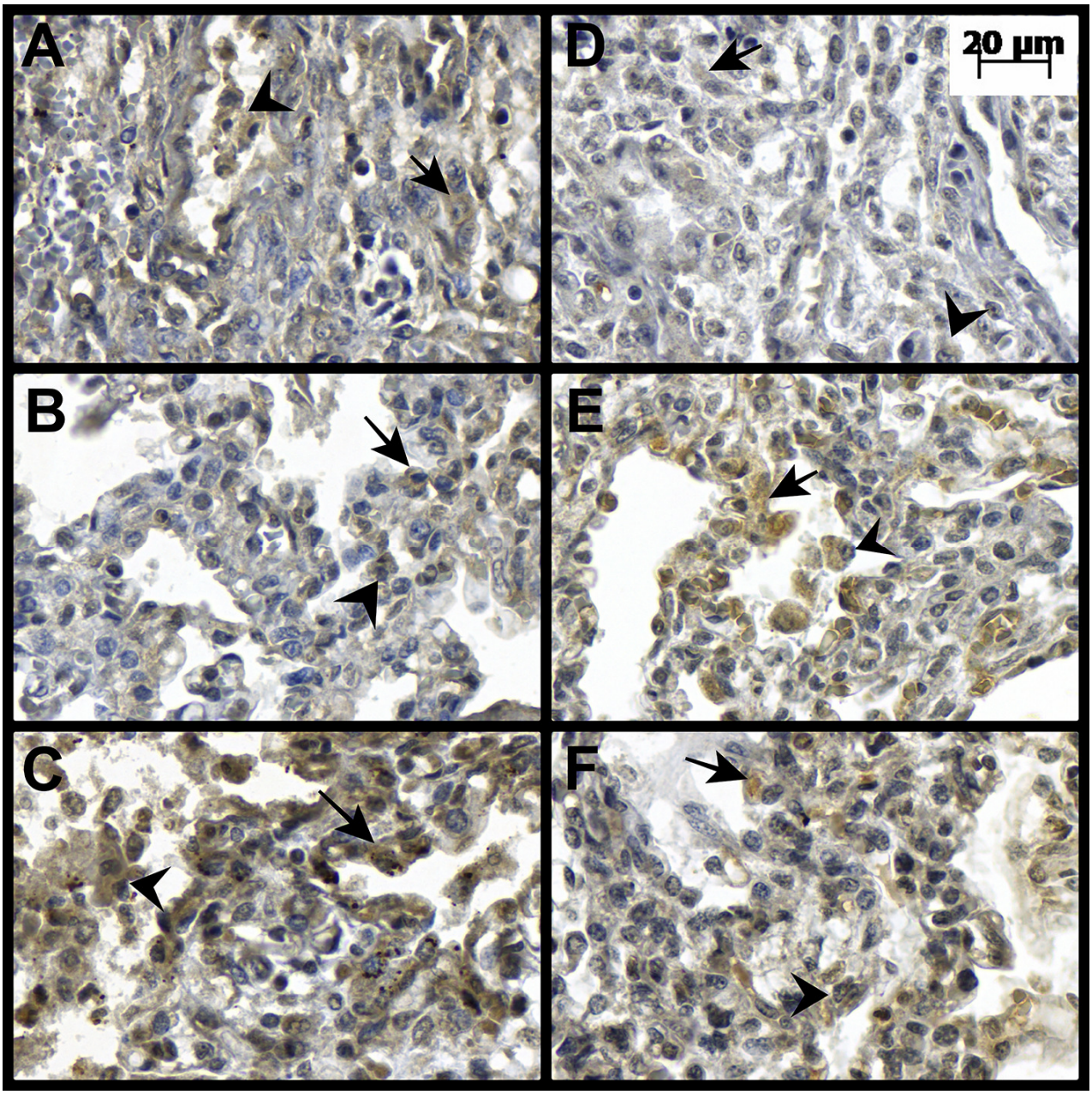

FIGURE 1

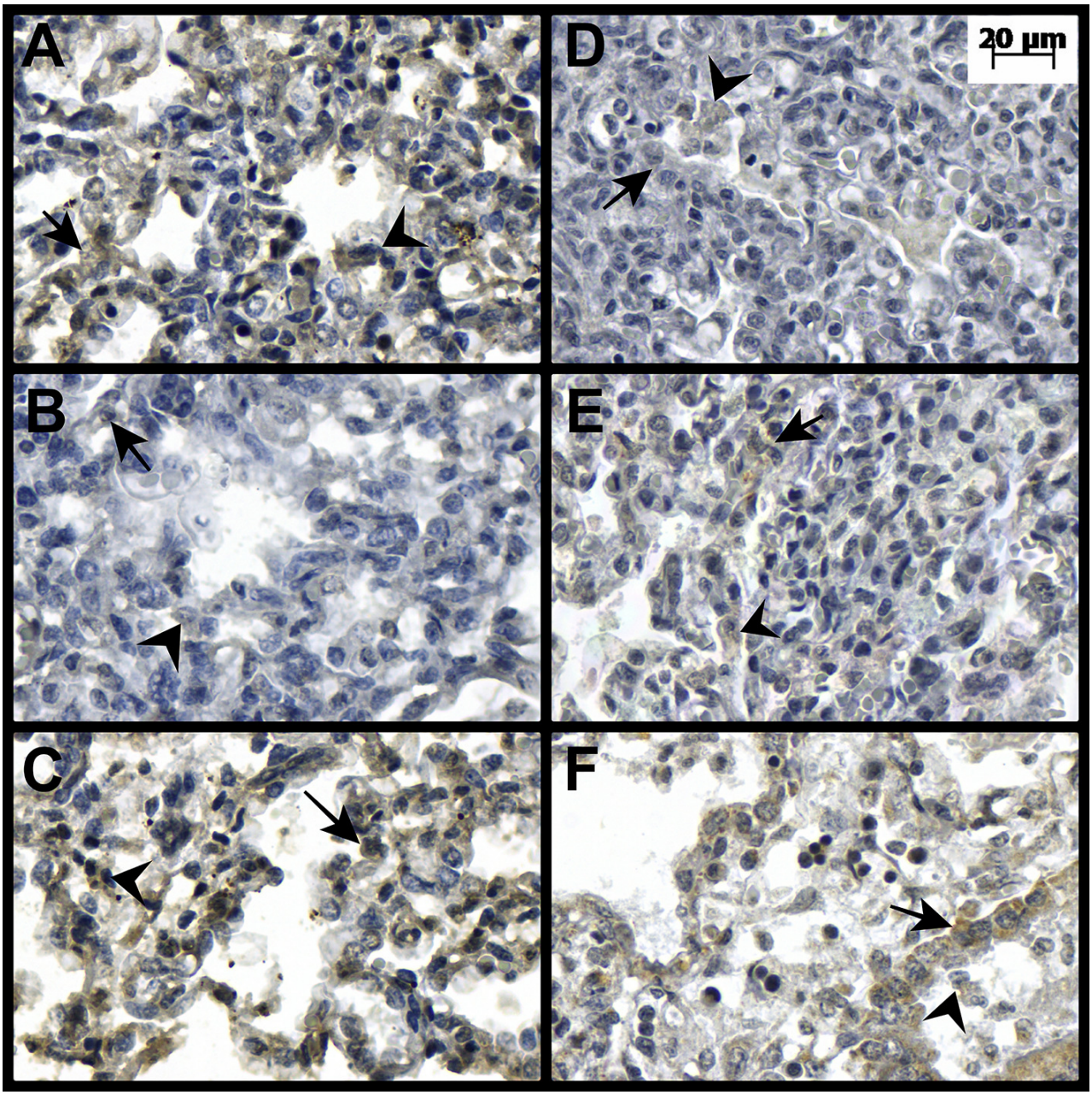

FIGURE 2

TABLE 1 - Results of the morphometric analysis performed to distinguish the group with new BPD from the group without BPD

Variables	Group	Mean ± SD	Median (Min-Max)	P value *
Perimeter (μm^2)	Without BPD (n=16)	560.4 ± 168.8	552.7 (360.8-1040.7)	0.548
	New BPD (n=5)	481.5 ± 182.4	446.9 (269.1-707.4)	
Alveoli number	Without BPD (n=16)	55.8 ± 15.2	58.5 (30.2-77.5)	0.050
	New BPD (n=5)	40.9 ± 15.1	39.6 (28.3-65.9)	

* Non-parametric Mann-Whitney test ($p < 0.05$); SD: Standard Deviation; Min: Minimum; Max: Maximum; BPD: Bronchopulmonary dysplasia.

TABLE 2 - The mean duration of exposure to oxygen and the mean survival time of each group

Variables	Total (n = 32)	Classic BPD (n = 11) †	New BPD (n = 5) †	Without BPD (n = 16) †	P value *
Duration of oxygen use (days)	9.7	21.4	10.4	1.4	**<0.001**
Survival time (days)	10.7	23.4	12.4	1.4	**<0.001**

* Non-parametric Mann-Whitney test ($p < 0.05$). † Mean; BPD: Bronchopulmonary dysplasia.

TABLE 3 – Base line characteristics

Variables	Classic BPD	New BPD	Without BPD	P value
Gender male †	5 (45.5)	1 (20.0)	5 (31.3)	**0.017** **
female †	6 (54.5)	4 (80.0)	11 (68.8)	
Gestational age (weeks) £	29.0 ± 2.6	32.0 ± 2.8	28.8 ± 2.6	0.092 §
Birth weight (grams) £	1021.4 ± 288.9	1340.0 ± 397.5	957.8 ± 357.5	0.132 §
Parkin score £	32.0 ± 3.3	33.4 ± 3.1	28.8 ± 2.6	**0.005** §
Apgar 1 £	2.6 ± 2.0	6.0 ± 3.5	3.5 ± 2.7	0.186 §
Apgar 5 £	6.2 ± 2.6	8.4 ± 1.9	5.0 ± 3.5	0.087 §

** Fisher's exact test (gender with Bonferroni-corrected - $p<0.017$); § Non-parametric Mann-Whitney test ($p < 0.05$); † number (percentage); £ Mean ± Standard Deviation; Apgar 1/5: Scores at 1 and 5 minutes after birth; BPD: bronchopulmonary dysplasia.

TABLE 4 – Prenatal and postnatal factors of the sample (medical record data)

Variables	Yes or No	Classic BPD	New BPD	Without BPD
Prenatal factors				
Premature ROM	**Yes**	1 (10.0)	1 (20.0)	4 (33.3)
	No	9 (90.0)	2 (80.0)	8 (66.7)
Clinical chorioamnionitis	**Yes**	1 (9.09)	0 (0.0)	2 (12.5)
	No	10 (90.9)	5 (100.0)	14 (87.5)
Gestational diabetes	**Yes**	0 (0.0)	1 (20.0)	0 (0.0)
	No	11 (100.0)	4 (80.0)	16 (100.0)
HDP	**Yes**	3 (27.3)	3 (60.0)	2 (13.3)
	No	8 (72.7)	2 (40.0)	13 (86.7)
Posnatal factors				
Necrotizing Enterocolitis *	**Yes**	7 (63.6)	2 (40.0)	1 (6.3)
	No	4 (36.4)	3 (60.0)	15 (93.8)
Pneumonia *	**Yes**	6 (54.6)	0 (0.0)	1 (6.3)
	No	5 (45.5)	5 (100.0)	15 (93.8)
PDA *	**Yes**	10 (90.9)	2 (40.0)	2 (15.5)
	No	1 (9.1)	3 (60.0)	14 (87.5)
Sepsis *	**Yes**	11 (100.0)	4 (80.0)	3 (18.8)
	No	0 (0.0)	1 (20.0)	13 (81.3)
Perinatal asphyxia	**Yes**	6 (54.6)	1 (20.0)	9 (56.3)
	No	5 (45.4)	4 (80.0)	7 (43.7)
Antibiotic use	**Yes**	10 (90.9)	4 (80.0)	8 (50.0)
	No	1 (9.1)	1 (20.0)	8 (50.0)
Pulmonary hemorrhage	**Yes**	3 (27.3)	1 (20.0)	1 (6.3)
	No	8 (72.7)	4 (80.0)	15 (93.7)
Pulmonary hypertension	**Yes**	2 (18.2)	1 (20.0)	0 (0.0)
	No	9 (81.8)	4 (80.0)	16 (100.0)

** Fisher's exact test (With Bonferroni-corrected - p<0.017); * Data with statistical significance (p > 0.017); † number (percentage); BPD: bronchopulmonary dysplasia; HDP: specific hypertensive disease of pregnancy; PDA: Patent ductus arteriosus.

TABLE 5 - Immunohistochemical analysis of interleukins 6, 8, 10 and 17 in pulmonary tissue samples of premature infants.

Interleukins	Group	Mean ± SD	Median (Min-Max)	P value *
IL-6	**Classic BPD (n=11)**	5836 ± 1658	5672 (3589-8192)	0.183
	New BPD (n=5)	5624 ± 1866	4542 (4037-8020)	
	Without BPD (n=16)	7049 ± 1369	7033 (3987-10036)	
IL-8	**Classic BPD (n=11)**	1281 ± 749	1019 (241-2705)	0.476
	New BPD (n=5)	1887 ± 791	1848 (713-2827)	
	Without BPD (n=16)	1790 ±1296	1414 (180-4007)	
IL-10	**Classic BPD (n=11)**	808 ± 652	503 (71-2085)	0.446
	New BPD (n=5)	531 ± 667	162 (73-1631)	
	Without BPD (n=16)	823 ± 1084	372 (25-4045)	
IL-17	**Classic BPD (n=11)**	886 ± 637	675 (239-2249)	**0.029 ****
	New BPD (n=5)	1382 ± 726	1443 (521-2224)	
	Without BPD (n=16)	1686 ± 747	1681 (486-3248)	

* Non-parametric Kruskal-Wallis test, p <0.05; SD: Standard Deviation; Min: Minimum; Max: Maximum; BPD: Bronchopulmonary dysplasia; ** significance between Classic *versus* Without groups.

5. CONCLUSÃO

5.1 CONCLUSÃO GERAL

Este estudo com tecido pulmonar de neonatos com DBP mostrou que as interleucinas 6, 8, 10 e 17 não parecem estar envolvidas na fisiopatogênese da DBP. Em contrapartida, a IL-17 apresentou maior expressão no grupo sem DBP, talvez sendo um fator de proteção para a DBP. Mais estudos são necessários para avaliar o papel das interleucinas como biomarcadores e até mesmo como fatores terapêuticos para a DBP.

5.2 CONCLUSÕES ESPECÍFICAS

Não houve correlação da expressão tecidual das interleucinas 6, 8,10 e 17 com os dados clínicos dos grupos, tanto nos dados pré-natais como nos pós-natais.

6. REFERÊNCIAS DA INTRODUÇÃO E REVISÃO DE LITERATURA

AHLFELD, S. K.; CONWAY, S. J. Aberrant signaling pathways os the lung mesenchyme and their contributions to the pathogenesis of bronchopulmonary dysplasia. **Birth Defects Res A ClinMolTeratol**, v. 94, n, 1, p. 3-15, 2012.

AMBALAVANAN, N.e col. Cytokines associeted with bronchopulmonary dysplasia or death in extremely low birth weight infants. **Pediatrics**, v. 123, n.4, p. 1132-1141, 2009.

AN, H.; NISHIMAKI, S.; OHYAMA, M. e col. Interleukin-6, interleukin-8 and soluble tumor necrosis factor receptor-I in the cord blood as predictors of chronic lung disease in premature infants. **Am J ObstetGynecol**, v. 191, n. 5, p. 1649-1654, 2004.

BAIER, J.R.; LOGGINS, J.; YANAMANDRA, K. IL-10, IL-6 and CD14 polymorphisms and sepsis outcome in ventilated very low birth weight infants. **BMC Medice**, v. 4, n. 10, 2006.

BANCALARI, E. Changes in the pathogenesis and prevention of chronic lung disease of prematury. **American Journal of Perinatology**, v. 18, n.1, p. 1-9, 2001.

BARALDI, E. FILLIPONE, M. Chronic lung disease after premature birth. **N Engl J Med**, v. 357, n. 19, p. 1946-1955, 2007.

BEN-ARI, J; MAKHOUL, I. R. e col. Cytokine response during hyperoxia: sequential production of pulmonary tumor necrosis factor and interleukin-6 in neonatal rats. **IMAJ**, v. 2, p. 365-369, 2000.

BHANDARI, A.; BHANDARI,V. Biomarkers in bronchopulmonary dysplasia. **Paediatr Respir Rev**. v. 14, n. 3, p. 173-179, 2013.

BHANDARI, A.; BHANDARI,V. Bronchopulmonary dysplasia an update. **Indiam J Pediatr**, v. 74, p. 73-77, 2007.

BHANDARI, A; CARROL, C.; BHANDARI, V. BPD Preterm birth: a model for chronic lung disease and a substrate for ARDS in childhood. **Front Pediatr**, v. 4, n. 60, p 1-7, 2016.

BHANDARI, V.; BIZZARRO, M. J.; SHETTY, A. e col. Familial and genetic susceptibility to major neonatal morbidities in preterm twins. **Pediatrics**, v. 117, p. 1901-1906, 2006.

BHANDARI, V.; GRUEN, J.R. The genetics of bronchopulmonary dysplasia. **SeminPerinatol**, v. 30, n. 4, p. 185-91, 2006

BHANDARY, A.; PANITCH, H. B. Pulmonary outcomes in bronchopulmonary dysplasia. **SeminPerinatol**, v. 30, n. 4, p. 219-226, 2006.

BOHRER, B.; SILVEIRA, R. C.; NETO, E. C.; PROCIANOY, R. S. Mechanical ventilation of newborns infant chages in plasma pro-and anti-inflammatory cytokines. **J Pediatr**, v.156, n. 1, p. 16-19, 2010.

BOUSSO, A. **Contribuição da biópsia pulmonar a céu aberto** na avaliação de pneumopatia aguda, sem etiologia definida, em Unidade de Terapia Intensiva Pediátrica. São Paulo, 1998. Mestrado em Pediatria-Departamento de Pediatria, Faculdade de Medicina da Universidade de São Paulo, 1998.

CARON, J. E.; LA PINE, T. R., AUGUSTINE, N. H.; MARTINS, T. B,; KUMÁNOVICS, A.; HILL, H. R. Severely depressed interleukin-17 production by human neonatal mononuclear cells. **Pediatr Res**. n.76, v. 6, p. 522-527, 2014.

CERNY, L.; TORDA, J. S.; REHAN, V. K. Prevention and treatment of bronchopulmonary dysplasia: contemporary status and future outlook. **Lung**, v. 186, n. 2, p. 75-89, 2008.

CHAKRABORTY, M.; MCGREAL, E.P.; WILLIAMS, A.; DAVIES, P.L; POWELL W, ET AL. Role of serine proteases in the regulation of interleukin-8_{77} during the development of bronchopulmonary dysplasia in preterm ventilated infants. **PLOS ONE.** V**.**9, N.12, 2014.

CHEN, J.; LIN, J.; LUO, H.; LI, M. EFFECTS OF HUMAN INTERLEUKIN-10 ON VENTILATOR-ASSOCIATED LUNG INJURY IN RATS. **Inflammation.** n. 22, 2018.

CHESS, P. R.; D`ANGIO, C. T.; PRYHUBER, G. S.; MANISCALCO, W. M. Pathogenesis of bronchopulmonary dysplasia. **Seminars Perinatology**, v. 30, n. 4, p. 171-178, 2006.

CHOI, C. W.; BEYOND, K. e col. Decrease expression of transforming growth factor-beta 1 in bronchoalveolar lavage cells of preterm infants with maternal chorioamnionitis. **J Korean Med Sci**, v. 23, n. 4, p. 609-615, 2008.

COALSON, J.J.; WINTER, V.; LEMOS, R. A. Deacreasealveolarization in baboon survivors with bronchopulmonary dysplasia. **Am J RespCrit Care Med**, v. 152, p. 640-646, 1995.

COALSON, J.J.; WINTER, V.; SILER-KHODR, T.; YODER, B. A.; Neonatal chronic lung disease in extremely immature baboons. **Am J Respir Care Med**, v. 160, p. 1333-1346, 1999.

CORDERO, L.; SANASES, M.; DEDHIYA, P.; AYERS, L. W. Purulence and gram- negative bacilli intra tracheal aspirates of mechanically ventilated very low birth weight infants. **J Perinatol**, v. 21, p. 376-381, 2001.

COUROCLI, X. I.; WELTY, S. E.; RAMSAY, P. L. Detection of microorganisms in tracheal aspirates of preterm infants by polymerase chain reaction: association of adenovirus infection with bronchopulmonary dysplasia. **Pediatr Res**, v. 47, p. 225-232, 2000.

CUA, D. J., TATO, C. M. Innate IL-17-producing cells: the sentinels of the immune system. **Nat Rev Immunol**.n.10, v.7,p.479-489, 2010

DAME, J.B.; JUUL, S.E. The distribution of receptors for the pro-inflammatory cytokines interleukin (IL-6 and IL-8) in the developing human fetus. **Early Hum Dev**, v. 58, p. 25-39, 2000.

DAVIDSON, D.; PATEL, H.; DEGOY, A. C.; GERSHKOVICH, I.; VANCUROVA, I.; MISKOLCI, V. Differential effect of exogenous interleukin-10 versus glucocorticoids on gene expression and pro-inflammatory cytokine relase by polymorphonuclear leukocytes and monocytes of the newly born. **Am J Transl Res**, v. 5, n. 1, p. 103-115, 2013.

DE DOOY, J.; IEVEN, M.; STEVENS, W.; DE CLERCK, L.; MAHIEU, L. High levels of CXCL8 in tracheal aspirate samples taken at birth are associated with adverse respiratory outcome only in preterm infants younger than 28 weeks gestation. **Pediatr Pulmonol**. v. 42, p. 193–203, 2007.

DE DOOY, J. J; MAHIEU, L. M.; VAN BEVER, H. P. The role of inflammation in the development of chronic lung disease in neonates. **Eur J Pediatr**, v. 160, n. 8, p. 457-463, 2001.

DEAKINS, K.M. Bronchopulmonary dysplasia. **Respiratory Care,** v. 54, n. 9, p.1252-62, 2009.

DIMES, M.O. White paper on preterm birth, the global and regional toll. **White plains: March of Dimes Foundation**, 2009.

EHRENKRANZ, R.A.; WALSH, M.C.; VOHR, B.R.; JOBE, A.H.;WRIGHT, L.L., FANAROFF, A.A. et al. Validação da Definição de Displasia Broncopulmonar do Consenso do Instituto Nacional de Saúde. **Pediatrics,** v.10,n.6, 413-422, 2006

FOGLI, L.K.; SUNDRUD, M.S.; GOEL, S.; BAIWA, S.; JENSE, K.;DERUDDER, E.; SUN, A.; COFFRE, M.;UYTTENHOUSE, C.; SNICK, J.V.; SUPPRIAN, M.S.; RAO,A.;GRUNIG,G.;DURBIN,J.; CASOLA, S.; RAJEWSKY,K.;KORALOV, S. B. T-Cell-derived IL-17 mediates epithelial changes in the airway and drives pulmonary neutrophilia. **J Immunol**, v.191, n.6, 2013.

FORGHANI, B.; DENNIS, J. Immunoenzymatic staining. In: SCHIMDT, N.J.;EMMONS, R.W. Procedures for Rickettsial and Chlamydial Infections. **American Public Health Association**, 1989, p135-156.

GARINGO, A.; TESORIERO, L.; CAYABYAB, R.; DURAND, M.; BLAHNIK, M.; SARDESAI, S.; RAMANATHAN, R.; JONES, C.; KWONG, K.; LI, C.; MINCO, P. Constitutive IL-10 expression by lung inflammatory cells and risk for bronchopulmonary dysplasia, **Pediatr Res**. v. 62, n. 2, p. 197-202, 2007.

GLEDITSCH, D. D.; SHORNICK, L.,P.; VAN STEENWINCKEL, J,; GRESSENS, P.; WEISERT, R. P.; KOENIG, J .M. Maternal inflammation modulates infant immune response patterns to viral lung challenge in a murine model. **Pediatr Res**. n. 76, v. 1,p. 33-40, 2014

GOMEZ, R.; ROMERO, R.; GHEZZI, F.; YOON, B. H.; MAZOR, M.; BERRY, S. M. The fetal inflamatory response syndrome. **Am J Obstet Gynecol**. v. 179, p. 195-202, 1998.

GOMEZ POMAR, E.; CONCINA, V.A.; SAMIDE, A.; WESTGATE, P.M.; BADA, H.S. Bronchopulmonary Dysplasia: Comparison Between the Two Most Used Diagnostic Criteria. **Z Front Pediatr.** v.12, n.6, p. 397, 2018.

GREENHOUGH, A.; PREMKUMAR, M.; PATEL, D. Ventilatory strategies for extremely premature infant. **PediatrAnaesthesia**, v. 18, n. 5, p. 371-377, 2008.

GRONECK, P.; SPEER, B.; OPPERMANN, M. e col. Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of right-risk preterm neonates. **Pediatrics**, v. 93, n. 5, p. 712-781, 1994.

HAPPEL, K. I.; ZHENG, M.; YOUNG, E.; QUINTON, L. J.; LOCKHART, E.; RAMSAY, A. J.; SHELLITO, J. E.; SCHURR, J. R.; BAGBY, G. J.; NELSON, S.; KOLLS, J. K. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to Klebsiella pneumoniae infection**. J Immunol**. n. 1, v.9, p.4432- 4436, 2003.

HAWWA, R. L.; HOKENSON, M. A.; WANG, Y.; HUANG, Z.; SHARMA, S.; SANCHEZ-ESTEBAN, J. Differential expression of MMP-2 and -9 and their inhibitor in fetal lung cells exposed to mechanical stretch: regulation by IL-10. **Lung**, v. 189, n. 4, p. 341-349, 2011.

HAWWA, R. L.; HOKENSON, M. A.; WANG, Y.; HUANG, Z.; SHARMA, S.; SANCHEZ-ESTEBAN, J. IL-10 inhibits inflammatory cytokines releases by fetal mouse lung fibroblasts exposed to mechanical strech. **PediatrPulmonol**, v. 46, n. 7, p. 640-649, 2011.

HEINRICH, P. C.; BEHRMANN, I.; HAAN, S.; HERMANNS, H. M.; MÜLLER-NEWEN, G.; SCHAPER, F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. **Biochem J**, v. 374, n. 15, p. 1-20, 2003.

HIKINO, S; OHGA, S.; KINJO, T.; OCHIAI, M; INOUE, H; HONJO, S.; IHARA, K.; OHSHIMA, K.; HARA, T. Tracheal aspirate gene expression in preterm newborn and development of bronchopulmonary dysplasia. **PediatrInt**, v. 54, n. 2, p. 208-214, 2012.

HSIAO, C.C.; CHANG, J. C.; TSAO, L. Y.; YANG, R.C.; CHEN, H. N.; LEE, C. H.; LIN, C. Y.; TSAI, Y. G. Correlates of elevated interleukin-6 and 8-hidroxy-2-deoxyguanosine levels in tracheal aspirates from very low birth weight infants who develop bronchopulmonary dysplasia. **Pediatrics and Neonatology**, v.58, p 63-69, 2017.

HUANG, W.; NA, L.; FIDEL, P. L.; SCHWARZENBERGER, P. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. **J Infect Dis**. n.190, v.3, p.624-631, 2004

HULSMANN, A.R. ANKER, J.N. Evolution and natural historyof chronic lung disease of prematurity. **Monaldi Arch Chest Dis**. v. 52, p. 272-277, 1997.

HUUSKO, J. M.; KARJALAINEN, M.K.; MAHLMAN, M.; HAATAJA, R.; KARI, M. A.; ANDERSSON, S.; TOLDI, G.; TAMMELA, O.; RAMET, M.; HALLMAN, M. A study of genes encoding cytokines (IL6, IL10, TNF), cytokines receptors (IL6R, IL6ST), and glucocorticoid receptor (NR3C1) and susceptibility to bronchopulmonary dysplasia. **BMC Medical Genetics**. v.15, p 120-129, 2014.

JENSEN, E.A.; SCHMIDT,B. Epidemiology of bronchopulmonary dysplasia. **Birth Defects Res A Clin Mol Teratol.** v.100, n.3,p145-157, 2014.

JOBE, A. H.; BACALARI, E. Bronchopulmonary dysplasia. **Am J RespirCrit Care Med**, v. 163, p. 1723 - 1729, 2001.

JOBE, A.; IKEGAMI, M. Mechanisms initiating lung injury in the preterm. **Early Hum Dev**, v. 53, p. 81-94,1998.

JOBE, A. H. Mechanisms of lung injury and bronchopulmonary dysplasia. **Am J Perinatol**, v. 33, n.11,p.1076-1078

JOBE, A. H. The new BPD: arrest of lung development. **Pediatri Res**, v.46, p. 641-643.

JOHNSON TJ, PATEL AL, JEGIER BJ, ENGSTROM JL, MEIER PP. Cost of morbidities in very low birth weight infants. **J Pediatr**, v.162, n.2, p.243–249, 2013

JONSSON, B.; LU, Y. H.; NOACK, G.; BRAUNER, A.; TULLUS, K. Downregulatory cytokines in tracheobronchial aspirate fluid from infants with chronic lung disease of prematurity. **ActaPaediatr**, v. 89, n. 11, p. 1375-1380, 2000.

JONSSON, B; TULLUS, K.; BRAUNER, A;LU, Y. H.; NOACK, G. Early increase of TNF-α and IL-6 in tracheobronchial aspirate fluid indicator of subsequent chronic lung disease in preterm infants. **Archives of Diseases in Childhood**, v. 77, p. 198-201, 1997

KALLAPUR, S; JOBE, A. Contribution of inflammation to lung injury and development. **Arch Dis Child Fetal Neonatal**, v. 91, p. 132-135, 2006.

KAWAGUCHI, M.; KOKUBU, F.; KUGA, H.; MATSUKURA, S.; HOSHINO, H.; IEKI, K.; IMAI, T.; ADACHI, M.; HUANG, S. K. Modulation of bronchial epithelial cells by IL-17. **J Allergy Clin Immunol**. N2001 Nov;108(5):804–9.

KINUGASA, T.; SAKAGUCHI, T.; GU. X; REINECKER, H. C. Claudins regulate the intestinal barrier in response to immune mediators. **Gastroenterology.** n. 118, v. 6, p. 1001-1011, 2000.

KINSELLA, J. L.; GREENOUGH, A.; ABMAN, S. Bronchopulmonary dysplasia. **Lancet**, v. 367, p. 1421-1431, 2006.

KISHIMOTO, T. Interleukin-6: discovery of pleiotropic cytokine. **Arthritis Res Ther**, v. 8, n. 2, 2006.

KUMAR, B.; DE SILVA, M.;VENTER, D.J.; ARMES, J.E. Tissuemicroarrays: a practicalguide. **Pathology**, v.36, n.4, 295-300, 2004

KUNZMANN, S.; COLLINS, J. J.; KUYPERS, E.; KRAMER, B. W. Thrown off balance: effect of antenatal inflammation on the developing lung and immune system. **Am J ObstetGynecol**, jan, 2013.

LAUGHON, M. M.; SMITH, P. B.; BOSE, C. Prevention of bronchopulmonary dysplasia. **Seminars in fetal and neonatal care**, v. 14, n. 6, p 374-382, 2009.

LAWRENCE, S.M.; RUOSS, J. L.; WYNN, J.L. IL-17 IN NEONATAL HEALTH AND DISEASE. **Am J reprod immunol**. n. 79, v.5, p.12800**,** 2018.

LEAL, M.C.; ESTEVES-PEREIRA, A. P.; NAKAMURA-PEREIRA, M; e col. Childbirth in Brazil. **Reproductive Health**. v. 13, n.3, p127, 2016.

LEE, H.S.; KIM, C.K. Effect of recombinant IL-10 on cultured fetal rat alveolar type II cells exposed to 65%-hiperoxia. **Respiratory Research**. v. 12, p. 68-83, 2011.

LI, T.J.; ZHAO, L.L.; QIU,J.; ZHANG, H.Y.; BAI, G. X.; CHEN, L. Interleukin-17 antagonist attenuates lung inflammation through inhibition of the erk1/2 and nf-κb pathway in lps-induced acute lung injury. **Mol med rep**. N. 16, ,v, 2, p.2225-2232, 2017.

LINDEN, A; LAAN, M; ANDERSON, G. P. Neutrophils, interleukin-17a and lung disease. **Eur respir j**. V.25, n.1, p 159-172, 2005.

LISTA, G.; CASTOLDI, F.; FONTANA, P.; REGGIANI, A.; BIANCHI, S.; COMPAGNONI, G. Lung inflammation in preterm infants with respiratory distress syndrome: effects of ventilation with different tidal volumes. **PediatrPulmonol**, v. 41, n. 4, p. 357-363, 2006.

LIU, D.Y.; WU, J.; ZHANG, X, Y., FENG, Z.C. Expression of IL-8, SP-A and TGF-beta1 in bronchoalveolar lavage fluid of neonates with bronchopulmonary dysplasia. **Zhongguo Dang Dai**, v. 12, n. 6, p. 444-446, 2010.

MASSARO, D.; MASSARO, G.D. Critical period for alveologenesis and early determinants of adult pulmonary disease. **Am J Physiol Lung cell MolPhysiol**, v. 287, p. 715-717, 2004.

MCCLINTOCK, J. K.; WAGNER, E. M. Role of IL-6 in systemic angiogenesis of the lung. **J ApplPhysiol**, v. 99, p. 861-866, 2005.

MCFARLANE, M.J.; FEINSTEIN, A.R.; WELLS, C.K.; CHAN, C. K. The epidemiologic necropsy: unexpected detections,demographic selections and changing rates of lung cancer. **JAMA**, v.258, p. 331-338, 1987.

MCGOWAN, E. C.; KOSTADINOV, S. e col. Placental IL-10 dysregulation and association with bronchopulmonary dysplasia risk. **Pediatr Res**, v. 66, n. 4, p. 455-460, 2009.

MIKACENIC, C.; HANSEN, E. E.; RADELLA, F.; GHARIB, S. A.; STAPLETON, R. D.; WURFEL, M. M. Interleukin-17A Is Associated With Alveolar Inflammation and Poor Outcomes in Acute Respiratory Distress Syndrome. **Crit Care Med**. n. 44, v. 3, p. 496-502, 2016.

MIRRO, R.; ARMSTEAD, W.; LEFFLER, C. Increased airway leukotriene levels in infants with severe bronchopulmonary dysplasia. **Am J Dis Child**, v. 144, p. 160-161, 1990.

MONTE, L.F.V.; SILVA FILHO, L.V.F.da; MYOSHI, M. H.; ROZOV, T. Displasia broncopulmonar. **J Pediatr**, v. 81, n. 2, p. 99-110, 2005.

MOSSER, D.M.; ZHANG, X. Interleukin-10: new perspectives on an old cytokine. **Immunol rev**. v. 226, p. 205-218, 2008.

MUKAIDA, N. Pathophysiological roles of interleukin 8/cxcl8 in pulmonary diseases. **Am J Physiol Lung Cell MolPhysiol**, v. 284, p. 566-577, 2003.

NATIONAL CENTER FOR HEALTH STATISTICS. Final Natality Data. 2016. Disponível em: www.marchofdimes.org/peristats

NORTHWAY, W. H.; ROSAN, R. G.; PORTER, D. Y. Pulmonary disease following respiratory therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. **N Engl J Med**, v. 276, n. 7, p. 357-368, 1967.

O´REILLY, M.; SOZO, F.; HARDING, R. The impact of preterm birth and bronchopulmonary dysplasia on the developing lung: long- term consequences for respiratory health. **ClinExpPharmacolPhysiol**, v. 16, n. 2, 2013.

PAANANEN, R; HUSA, A.K.; VUOLTEENAHO, R.;HERVA, R.;KAUKOLA, T.; HALLMAN, M. Blood cytokines during the perinatal period in very preterm infants: relationship of inflammatory response and bronchopulmonary dysplasia. **J Pediatr**, v.154, n.1, 2009.

PARERA, M. C. E e col. Distal angiogenesis: a new concept for lung vascular morphogenesis. **Am J Physiol Lung Cell MolPhysiol**, v.288, p. 141-149, 2005.

PARSONS, P. E, EISNER, M. D.; THOMPSON, B.T. E COL. Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury. **Crit Care Med**, v. 33, p. 1-6, 2005.

PIERCE, M. R.; BANCALARI, E. The role of inflammation in the pathogenesis of bronchopulmonary dysplasia. **PediatrPulmonol**, v. 18, p. 371-378, 1995.

PURISCH, S. E.; GYAMFI-BANNERMAN, C. Epidemiology of preterm birth. **Seminars in Perinatology**, v. 41, p. 387-391, 2017.

PURSEY, V.A.; MACPHERSON, R.I.; CHERNICK, V. Pulmonary fibroplasia following prolonged artificial ventilation of newborn infant. **Can Med Assoc J**, v. 100, n. 10, p. 451-457, 1969.

RAJITA, P.; CARROZZI, V. R.; SAMBANDAM, A. The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases. **Immunology**, v. 134, n. 1, p. 8-16, 2011.

RAMANATHAN, V. Infection and preterm birth: evidence of common causal relationship with bronchopulmonary dysplasia and cerebralpalsy. **J Paediatr Child Health**, v. 36, n. 4, p. 293-296, 2001.

REDE BRASILEIRA DE PESQUISAS NEONATAIS. Perfil dos Recém-Nascidos com PN < 1500g que nasceram e permaneceram em cuidado no mesmo hospital. 2015. Disponível em: www.redeneonatal.fiocruz.br

RIGHETTI, R. F.; SANTOS, T. M.; CAMARGO, L. M.; ARISTÓTELES, L. C R.C. R. B; FUKUZAKI, S.; SOUZA, F. C. R.; SANTANA, F. P.R.; AGRELA, M. V. R; CRUZ, M. M; VALE, M. I.C.A.; GENARO, I. S.; ROMANHOLO, B. M.S.; LEICK, E.A.; MARTINS, M.A.; PRADO, C.; TIBERIO, A.F.L.C. Protective effects of anti-il17 on acute lung injury induced by lps in mice. **Front Pharmacol.** n. 9, p. 1021, 2018.

RITO, D. C.; VIEHL, L.T.; BUCHANAN, P. M; HARIDAS, S.; KOENIG, J. M. Augmented Th17-type immune responses in preterm neonates exposed to histologic chorioamnionitis **PediatriRes**. n. 81, v. 4, p.639-645, 2017.

ROCHA, G.; PROENÇA, E.; GUEDES, A. e col. Cord blood levels of IL-6, IL-8 and IL-10 may be early predictors of bronchopulmonary dysplasia in preterm newborns small for gestacional age. **Dis Markers**, v. 33, n. 1, p. 51-60, 2012.

RYAN, R. M.; AHMEQ, Q.; LAKSHMINRUSIMHA, S. Inflammatory mediators in the immunobiology of bronchopulmonary dysplasia. **Clin Rev Allergy Immunol**, v. 34, n. 2, p. 174-190, 2008.

SAKAGUCHI, R.; CHIKUMA, S.; SHICHITA, T.; MORITA, R.; SEKIYA, T.; OUYANG, W.; ET AL. Innate-like function of memory Th17 cells for enhancing endotoxin-induced acute lung inflammation through IL-22. **Int. Immunol.** n.28, p. 233–243, 2016

SCHULTZ, C.; TEMMING, P.; BUCSKY, P.; GOPEL, W.; STRUNK, T.; HATEL, C. Immature anti-inflammatory response in neonates. **ClinExpimmunol**, v. 135, p. 130-136, 2004.

SHENNAN, A.T.; DUNN, M.S.; OHLSSON, A.; LENNOX, K.; HOSKINS, E. M. Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. **Pediatrics**, v. 82, n. 4, p. 527-532, 1988.

SHIMOYA, K.; TANIGUCHI, T.; MATSUZAKI, N.; MORIYAMA, A.; MURAT, Y.; KITAJIMA, H.; FUJIMURA, M.; NAKAYAMA, M. Chorioamminionitis decrease incidence of respiratorydistress syndrome by elevanting fetal interleukin-6serum concentration. **Hum reprod**, v. 15, p. 2234-2240, 2000.

SILVA, C. N.; SANTOS, M.; BAPTISTA, M. J.; MOURA, R. S; PINTO, C. IL-6 is constitutively expressed during lung morphogenesis and enhances fetal lung explant branching. **Pediatr Res**, v. 60, p. 30-36, 2006.

SIMON, R.; MIRTACHER, M.;SAUTER, G. Tissue microarrays. **Biotechniques**, v.36, p.98-105, 2004.

SMOLEN, J. S.; MAINI, R. N.; Interleukin-6: a new therapeutic target. **Arthritis Res Ther**, v. 8, n. 2, 2006.

SOOD, B. G.; SHANKARAN, S.; SCHELONKA, R. L.; SAHA, S.; BENJAMIN, D. K. JR; SÁNCHEZ, P. J.; ADAMS-CHAPMAN, I.; STOLL, B. J.; THORSEN, P.; SKOGSTRAND, K.; EHRENKRANZ, R. A.; HOUGAARD, D. M.; GOLDBERG, R. N.; TYSON, J. E.; DAS, A.; HIGGINS, R. D.; CARLO, W. A. Cytokine profiles of preterm neonates with fungal and bacterial sepsis. **Pediatr res**. n.72, v.2, p.212-220, 2012.

SPEER, C. Inflammation and bronchopulmonary dysplasia: a continuing story. **Semin Fetal Neonatal**, v. 11, p. 354-362, 2006.

SPEER, C. P. Inflammation and bronchopulmonary dysplasia. **SeminNeonatol**, v. 8, n. 1, p. 29-38, 2003.

SPEER, C. P. Pre-and postnatal inflammatory mechanisms in chronic lung disease of preterm infants. **PediatrResp Reviews**, v. 5, p. 241-244, 2004.

SU,B.H.; CHIU, H.Y.; LIN, T. W.; LIN, H.C. Interleukin-8 in bronchoalveolar lavage fluido f premature infants at risk of chronic lung disease. **J Formos Med Assoc**, v.104, n. 4, p. 244-248, 2005.

TAPIA, J. L.; AGOST, D.; ALEGRIA, A.; STANDEN, J. e col Bronchopulmonary dysplasia: incidence, risk factors and resource utilization in a population of South American very low birth weight infants. **J Pediatr**, v. 82, n. 1, p. 15-20, 2006.

TANNURI, U; FREDDI, N. A. Displasia Broncopulmonar. In: CARVALHO, W. B.; HIRSCHHEIMER, M. R.; PROENÇA, J. O. F.; FREDDI, N.A.; TROSTER, E. J. **Ventilação Pulmonar mecânica em pediatria e neonatologia**. São Paulo: EditoraAtheneu, 2004, p. 559-566.

THOMPSON, A.; BHANDARI, V. Pulmonary biomarkers of bronchopulmonary dysplasia. **Biomarker Insights**, v. 3, p. 361-373, 2008.

TSAI, H,C.; VELICHKO, S.; HUNG, L. Y.; WU, R. IL-17A and Th17 cells in lung inflammation: an update on the role of Th17 cell differentiation and IL-17R signaling in host defense against infection. **Clin Dev Immunol**. 2013

VON BISMARCK, P.; CLASS, A.; SCHICKOR, C.; KRAUSE, M. F.; ROSE-JOHN, S. Altered pulmonary interleukin-6 signaling in preterm infants developing bronchopulmonary dysplasia. **Exp Lung Res**, v.34, n. 10, p. 694-706, 2008.

WARD, N. S.; WAXMAN, A. B.; HOMER, R. J.; MANTELL, L.L.; EINARSSON, O.; DU,Y.; et al. Interleukin -6 induced protection in hiperoxic acute lung injury. **Am j Respir Cell Mol Bio**, v. 22, p. 535-542, 2000.

XING, Z.; GAULDIE, J.; COX, G.; BAUMANN, H.; JORDANA, M.IL-6 ia an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory response. **J Clin Invest**, v. 101, p. 311-320, 1998.

YAMAGATA, T.; ICHINOSE, M. Agents against, cytokine synthesis or receptors. **Eur J Pharmacol**, v. 533, p. 289-301, 2006.

YOU, Q.H.; SUN, G.Y.; SHEN, J. L.; WANG, Y. Interleukin-17F-induced pulmonary microvascular endothelial monolayer hypermeability via the protein kinase C pathway. **Surg Res**, v. 162, n. 1, p. 110-121, 2010.