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Campus de Botucatu

PG-BGA

Avaliação do inflamassoma NLRP3 e autofagia em placentas de gestantes portadoras de pré-eclâmpsia

INGRID CRISTINA WEEL

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia Celular Estrutural e Funcional.*

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SUMÁRIO

"Agradeço todas as dificuldades que enfrentei; não fosse por elas, eu não teria saído do lugar. As facilidades nos impedem de caminhar".

-Chico Xavier-

Resumo

A pré-eclâmpsia (PE) é uma síndrome clinicamente identificada por hipertensão arterial e proteinúria e está associada à produção excessiva de citocinas proinflamatórias, deficiência na produção de citocinas reguladoras e aumento nos níveis séricos de anticorpos antifosfolípides (aPLs) em pacientes com PE grave. Os aPLs são uma família de autoanticorpos que reagem com proteínas ligantes de fosfolipídios, sendo seu principal alvo a beta-2 glicoproteina I (β2GPI). Estes anticorpos são responsáveis por inibir a diferenciação do sinciciotrofoblasto e restringir a migração trofoblástica, resultando em remodelação anormal das arteríolas espiraladas, alteração característica da PE. O inflamassoma é um complexo proteico que promove a secreção das citocinas proinflamatórias interleucina-1 beta (IL-1β) e interleucina 18 (IL-18) e, também a secreção da proteína "high-mobility group box 1" (HMGB1) após ativação por patógenos e/ou produtos endógenos associados ao dano celular. A autofagia é uma via de degradação celular ou de eliminação de organelas e proteínas através de processos lisossomais que são importantes para a manutenção da homeostase celular, promovendo a sobrevivência das células em resposta ao estresse. O presente trabalho teve como objetivos: 1. Investigar as proteinas relacionadas ao inflamassoma e à autofagia em placenta de gestantes portadoras de pré-eclâmpsia e de normotensas; 2. Avaliar a relação existente entre inflamassoma e autofagia em células de trofoblasto extraviloso humano (SW.71), induzida por aPL. Fragmentos placentários foram coletados de 20 gestantes normotensas e de 20 gestantes com pré-eclâmpsia para avaliar a expressão gênica e proteica de NLRP3, caspase-1, IL-1β, fator de necrose tumoral-alpha (TNF-α), IL-18, HMGB1, proteína de cadeia leve (LC3B-II), beclin-1 e de "mammalian target of rapamycin" (mTOR). As respostas autofágica e inflamatória e, a expressão de fatores antiangiogênicos também foram analizadas em células SW.71 induzidas por aPL. Os resultados mostraram níveis elevados de RNAm de NLRP3, caspase-1, IL-1β, TNF-α e HMGB1 em tecido placentário, bem como aumento significativo dos níveis de caspase-1, IL-1β, TNF-α e HMGB1 em homogenato de placenta de gestantes portadoras de PE quando comparadas às normotensas. A expressão gênica e proteica de IL-18 mostrou-se significativamente diminuida no grupo de gestantes pré-eclâmpticas. A expressão das proteínas relacionadas à autofagia, LC3B-II e beclin-1 e os níveis de RNAm destas proteinas mostraram-se significativamente diminuídos em placenta de gestantes com PE comparados aos de gestantes normotensas. Por outro lado, o RNAm e a proteína de mTOR foram mais elevados nas gestantes com PE em comparação com as normotensas. Nas células SW.71 observou-se que o tratamento com aPL por 8h induziu diminuição significativa da razão LC3B-II/LC3BI seguida de aumento significativo após

72 hr do estímulo. Esses resultados não foram observados na expressão de beclin-1 e p62. Em cultura das células submetidas à inibição do processo autofágico detectou-se aumento na secreção de IL-1β, IL-8 e endoglina solúvel (sEng), enquanto nas culturas em que a autofagia não foi inibida observou-se diminuição da secreção de sEng e de fms-like tyrosine-kinase-1 solúvel (sFlt-1). Além disso, a permanência do processo autofágico reduziu significativamente a secreção de IL-1β e IL-8 em células induzidas por aPL. Em conclusão, os resultados obtidos mostram que placenta de gestantes com PE apresentam ativação do inflamassoma NLRP3 e diminuição da resposta autofágica, sugerindo que estes processos podem contribuir para a patogênese da PE. O estímulo de células de trofoblasto extraviloso com aPL é capaz de diminuir a resposta autofágica nessas células por meio de ativação do inflamassoma. O restabelecimento tardio da autofagia nessas células poderia ocorrer, provavelmente, devido à tentativa desse processo em controlar a ativação do inflamassoma.

Palavras Chave: autofagia, inflamassoma, placenta, pré-eclâmpsia e síndrome anti-fosfolípide.

Abstract

Preeclampsia (PE) is a syndrome clinically identified by hypertension and proteinuria and is associated with excessive production of proinflammatory cytokines, deficiency in the production of regulatory cytokines, and increased serum levels of antiphospholipid antibodies (aPLs) in patients with severe forms of PE. aPLs are a family of autoantibodies that react with phospholipid binding proteins, which the main target is beta 2 glycoprotein-I (β2GPI). These antibodies are responsible for inhibiting the differentiation of syncytiotrophoblast and restrict trophoblast migration, resulting in abnormal remodeling of the spiral arterioles, characteristic alteration in PE. The inflammasome is a protein complex that promotes the secretion of the proinflammatory cytokines interleukin-1 beta (IL-1β) and interleukin 18 (IL-18), and also the secretion of high-mobility group box 1 (HMGB1) protein after activation by pathogens and/or endogenous products associated with cellular damage. Autophagy is a pathway of cell degradation or elimination of organelles and proteins by lysosomal processes that are important for the maintenance of cellular homeostasis by promoting the survival of cells in response to stress. The objectives of the present study are: 1. To investigate the proteins related to inflammasome and autophagy in placenta from pregnant women with PE and from normotensive control; 2. To evaluate the relationship between inflammasome and autophagy in human extravillous trophoblast cells (SW.71) induced by aPL. Placental fragments were collected from 20 normotensive pregnant women and from 20 pregnant women with PE to evaluate gene and protein expression of NLRP3, caspase-1, IL-1β, tumor necrosis factor-alpha (TNF-α), IL-18, HMGB1, light chain protein (LC3B- II), beclin-1 and mammalian target of rapamycin (mTOR). The autophagic and inflammatory responses as well as the expression of antiangiogenic factors were also analyzed in SW.71 cells induced by aPL. The results showed higher levels of mRNA for NLRP3, caspase-1, IL-1β, TNF-α and HMGB1 in placental tissue as well as significant increase in caspase-1, IL-1β, TNF-α and HMGB1 levels in placental homogenate from women with PE compared with the normotensive group. Gene and protein expression of IL-18 was significantly decreased in the group of preeclamptic women. The expression of proteins related to autophagy, LC3B-II and beclin-1 and the mRNA levels of these proteins were significantly decreased in placenta of women with PE compared with the normotensive ones. On the other hand, mRNA and protein for mTOR were higher in women with PE. In SW.71 cells it was observed that the treatment with aPL for 8h induced significant decrease in LC3B-II/LC3BI ratio, followed by its significant increase after 72hr of aPL stimulation. These results were not observed in the expression of beclin-1 and p62. When SW.71 cells were

submitted to autophagy inhibition a significant increase in IL-1β, IL-8 and soluble endoglin (sEng) was detected, whereas the maintenance of autophagy lead to reduced secretion of sEng and fms-like tyrosine kinase-1-soluble (sFlt-1). Furthermore, the maintenance of the autophagic process significantly reduced the secretion of IL-1β and IL-8 in cells induced by aPL. In conclusion, the results demonstrated that placenta from preeclamptic women shows NLRP3 inflammasome activation and decreased autophagic response suggesting that these processes may contribute to the pathogenesis of PE. The stimulus of extravillous trophoblast cells with aPL caused decrease in the autophagic response in these cells by inflammasome activation, leading to delayed recovery of autophagy, which may probably occur due to an attempted of inflammasome control by the autophagy process.

Keywords: autophagy, inflammasome, placenta, preeclampsia, antiphospholipid syndrome.

Introdução

1. Introdução

1.1. Pré-eclâmpsia, síndrome antifosfolípide e anticorpos antifosfolípides

A pré-eclâmpsia (PE) é uma síndrome que incide entre 2% e 8% das gestações e tem sérios impactos na saúde materna e fetal (Kane et al., 2014). É uma doença sistêmica, caracterizada por múltiplas alterações no organismo materno e clinicamente identificada por hipertensão arterial e proteinúria, que se manifestam após a 20ª semana de gestação (NHBPEP, 2000; De Oliveira et al., 2010).

Embora esta síndrome não esteja completamente compreendida, há relatos de que sua fisiopatologia é influenciada tanto por fatores maternos como placentários (Robillard, 2002; Redman & Sargent, 2005). Fundamentada nesse conceito, a literatura sugere a interação de vários mecanismos responsáveis pela característica multisistêmica da doença, tais como: placentação inadequada (Pijnenborg et al., 1983; Royle et al., 2009), disfunção endotelial (Khan et al., 2005), angiogênese insuficiente (Levine et al., 2006), má adaptação imunológica (Dekker & Sibai, 1999), estresse oxidativo (Gupta et al., 2005; Redman & Sargent, 2000; 2005) e resposta inflamatória excessiva (Redman et al., 1999; Redman & Sargent, 2003).

Durante a placentação normal, as arteríolas espiraladas maternas são invadidas pelo trofoblasto, resultando em remodelação da arteríola espiralada e assegurando um suprimento sangüíneo adequado para a placenta e o feto. No entanto, em placentas de gestantes com PE o trofoblasto não induz invasão adequada, gerando defeitos na remodelação da arteriola espiralada e resultando em má perfusão (Brosens et al., 1972; George & Granger, 2011). Roberts e Hubel (1999) sugerem que a PE tem seu início devido ao baixo fluxo sanguíneo uteroplacentário que resulta em hipóxia placentária. Portanto, a placenta tem papel essencial nessa doença, pois problemas na implantação e no

processo de placentação culminam em redução da perfusão sanguínea e, portanto, em hipóxia/isquemia placentária (Amash et al., 2007; Gilbert et al., 2008), que também pode causar restrição de crescimento fetal intrauterino (Hung et al., 2004).

Assim, a PE parece ser resultante de uma resposta inflamatória materna exacerbada, que inclui ativação de células inflamatórias e células endoteliais (Schuiling et al., 1997; Redman et al., 1999; Borzychowski et al., 2006). Segundo a literatura, essa patologia é caracterizada por produção excessiva de citocinas pró-inflamatórias, como o fator de necrose tumoral alpha (TNF-α) (Johnson et al., 2002; Redman & Sargent, 2003; Luppi & Deloia, 2006; Peraçoli et al., 2007) e quimiocinas (Visser al., 2007), bem como por alterações na produção de citocinas reguladoras (Pestka et al., 2004; Peraçoli et al., 2008; 2013; Weel et al.,2016). Além disso, é descrito que altos níveis de ácido úrico no plasma de pacientes com PE podem estar relacionados com a patogênese da PE por promover a resposta inflamatória (Bainbridge & Roberts, 2008). Dados da literatura, também revelam que em pacientes com PE grave, com idade gestacional menor que 34 semanas, observa-se um aumento de 20% no nível de anticorpos antifosfolípides (aPLs) quando comparadas as gestantes que não tiveram complicações na gestação e no parto (Heilmann et al., 2011).

Os aPLs são uma familia de auto anticorpos que reagem com proteínas ligantes de fosfolipídios, sendo o principal alvo a beta 2 glicoproetina I (β2GPI) que é constitutivamente expressa em trofoblasto placentário, especialmente em populações de trofoblasto extraviloso que são responsáveis pela invasão da decídua e remodelação da arteriola uterina espiralada (McNeil et al., 1990; Chamley et al., 1999; Quenby et al., 2005). Estes anticorpos são responsáveis por inibir a diferenciação do sinciciotrofoblasto (di Simone et al., 2005; D'Ippolito et al., 2014) e restringir a migração trofoblástica, resultando em remodelação anormal das arteríolas espiraladas (Mulla et al., 2010), visto que os alvos são as β2GPI expressas em trofoblastos placentários.

Esses aPLs são característicos da síndrome antifosfolípide (SAF) que é definida como uma doença autoimune, caracterizada pela presença de trombose, aborto recorrente e níveis elevados de aPLs (Hughes, 1983), os quais foram descritos por estarem relacionados com a prevalência e gravidade da síndrome HELLP (hemólise, níveis elevados de enzimas hepáticas e baixa quantidade de plaquetas) (Pauzner et al., 2003; Le Thi Thuong et al., 2005), porém pouco se sabe sobre o envolvimento do aPL na patogênese da PE e da síndrome HELLP. Alguns critérios de diagnóstico para a identificação de mulheres com SAF estão descritos na literatura e que incluem diagnóstico clínico e laboratorial. Os critérios clínicos incluem aborto recorrente antes da 10ª semana de gestação, obito fetal após a 10ª semana de gestação e histórico de trombose venosa ou arterial na presença ou ausência da gestação (de Jesus et al., 2014). Os critérios laboratorias incluem a identificação da presença de anticorpos anticoagulante lúpico, assim como aPL (anticorpos IgG/IgM, anti-β2GPI) e anticorpos anti-cardiolipina (Miyakis et al., 2006; Heilmann et al., 2011).

Em pacientes com SAF, as complicações da gestação estão associadas à inflamação na interface materno-fetal (Berman et al., 2005; Girardi et al., 2003; Mulla et al., 2009) e insuficiência placentária associada com redução de invasão trofoblástica (Alvarez et al.,2015; Mulla et al., 2010; Sebire et al., 2002). Estes achados clínicos são comprovados por estudos *in vitro* que descrevem a alteração na função celular de trofoblastos de primeiro trimestre quando β_2GPI é reconhecido pelo aPL. Essas alterações causadas são altamente relevantes, pois induzem nas células trofoblásticas a produção de níveis elevados de citocinas pró-inflamatórias como IL-1β e Interleucina 8 (IL-8) (Mulla et al., 2009; Mulla et al., 2013); ocorrendo, também, inibição da migração espontânea do trofoblasto (Mulla et al., 2010; Ulrich et al., 2015); e aumento na secreção do fator anti-angiogênico, a endoglina solúvel (sEng) (Carroll et al., 2011).

De modo geral, estas duas sindromes, PE e SAF, estão relacionadas com uma resposta inflamatória exacerbada e o desenvolvimento destas doenças é altamente prejudicial tanto para mãe quanto para o feto. Desta forma, estudos para a melhor compreensão destas síndromes são fundamentais para desvendar os mecanismos que envolvem o seu aparecimento.

1.2. Inflamassoma

Células do sistema imune inato, como monócitos, macrófagos e células dendríticas, expressam receptores conhecidos como receptores de reconhecimento de padrão (PRRs) considerados componentes centrais do sistema imune inato e envolvidos na resposta inflamatória de monócitos (Kim et al., 2005; Mazouni et al., 2008). Entre os vários PRRs, a principal família é denominada de receptores semelhantes ao *Toll* (TLRs), que reconhecem e ligam moléculas presentes na superfície de patógenos, conhecidas como padrões moleculares associados a patógenos (PAMPs) (Koga & Mor, 2008) e, também se ligam a produtos endógenos de células do hospedeiro, que são liberados durante lesão tecidual e denominados "sinais de perigo" ou padrões moleculares associados a danos (DAMPs) (Matzinger, 2002; Kim et al., 2005). Estes produtos são representados por moléculas como reativos intermediários do oxigênio (ROS), proteínas liberadas de células mortas (Park et al., 2004) e produtos liberados da matriz extracelular, como a fibronectina (Okamura et al., 2001).

Outros receptores PRRs compreendem os membros da família de receptores Nod-like ou NLR (proteína contendo domínio de oligomerização nucleotídica), que são proteínas citosólicas que reconhecem PAMP e DAMP citoplasmáticos e recrutam outras proteínas, formando complexos de sinalização que promovem a inflamação. Esses complexos de sinalização são chamados inflamassomos (Kawamura et al., 2014), que promovem a secreção de citocinas proinflamatórias, IL-1β e Interleucina 18 (IL-18), e também a secreção da proteina "high-mobility group box 1" (HMGB1) (Schroder & Tschopp, 2010; Gross et al., 2011). Ao contrário dos receptores TLR, que reconhecem PAMPs na superfície celular ou dentro dos endossomas, os receptores NLRs reconhecem moléculas microbianas ou endógenas dentro do citoplasma da célula hospedeira (Franchi et al., 2009). A ligação de LPS, ácido lipoteicóico, flagelina, cristais de ácido úrico, entre

outros PAMPs e DAMPs ativam receptores NLR, incluindo NLRP3, que são responsáveis pela ativação do inflamassomo (Lamkanfi et al., 2007). Após interação com o ligante, diversas proteínas NLRP3 idênticas formam um oligômero e cada NLRP3 do oligômero se liga à forma precursora inativa da enzima caspase-1 que se torna ativa e cliva a pró-IL-1β, resultando na geração de IL-1β biologicamente ativa que, subsequentemente, é secretada no meio extracelular (Pétrilli et al., 2007; Eder, 2009;. Franchi et al., 2009 ; Guo et al., 2015).

O HMGB1 foi inicialmente descrito como uma proteína nuclear presente em quase todos os tipos celulares (Yang et al., 2013). Quando o HMGB1 é liberado para o meio extracelular após o processo necrótico celular, ele age como mediador inflamatório (Chen et al., 2016), sendo descrito que pode contribuir para o desenvolvimento da resposta inflamatória em pacientes com PE (Zhu et al., 2015). Além disso, tem sido sugerido que o HMGB1 pode estar envolvido na patogênese da PE, devido sua elevada expressão no sinciotrofoblasto e no soro materno de gestantes com PE grave (Zhu et al., 2015).

Na PE os estudos demonstrando o papel do inflamassoma na interface materno-fetal são escassos. O envolvimento do inflamassoma NLRP3 na defesa imunológica placentária foi descrito por Pontillo et al. (2013). A estimulação de células placentárias como citotrofoblasto de primeiro trimestre e células da decídua humanas com LPS levaram a um aumento na expressão de caspase-1, IL-1β e NLRP3. A secreção de IL-1β por membranas fetais e por células mononucleares isoladas de cordão umbilical, da placenta e do sangue materno, obtidas de gestação de termo e estimuladas com LPS é dependente de caspase-1, sugerindo que o inflamassoma NLRP3 parece ser necessário para o processamento de IL-1β em tecido placentário (Maneta et al., 2014). Além disso, Mulla et al. (2011) demonstraram que células trofoblásticas de primeiro trimestre estimuladas com urato monossódico induzem a secreção de IL-1β. Segundo os autores, cristais de ácido

úrico podem ativar o inflamassomo no trofoblasto, sendo este um novo mecanismo de indução da inflamação na interface materno-fetal, que leva à disfunção placentária e a resultados adversos, como a PE.

Em trabalho recente, demonstramos que a expressão de RNAm para NLRP3, caspase-1, IL-1β e TNF-α por monócitos estimulados ou não com urato monosódico (MSU) mostrou-se significativamente aumentado em gestantes portadoras de PE quando comparadas às normotensas. Além disso, os monócitos de gestantes pré-eclâmpticas produziram níveis endógenos mais elevados de IL-1β e TNF-α, sugerindo que o ácido úrico desempenha papel importante na ativação do inflamassoma NLRP3, contribuindo para o desenvolvimento da patogênese da PE (Matias et al., 2015).

Em vista desses resultados e sabendo que a placenta tem papel fundamental para o desenvolvimento da PE, estudos da ativação do inflamassoma em placenta de gestantes com PE são necessários para a melhor compreensão dos mecanismos envolvidos na PE.

1.3. Autofagia

A autofagia refere-se à via celular de degradação ou eliminação de organelas danificadas e agregados proteicos através de processos lisossomais (Czaja et al., 2013; Ni et al., 2014), mantendo a homeostase celular e promovendo a sobrevivência da célula durante situações de estresse (Hung et al., 2013). Esse processo de autofagia é dependente da inibição do "mammalian target of rapamycin" (mTOR) pela privação de nutrientes. Quando o mTOR apresenta-se em elevadas quantidades é descrito como atenuador do processo autofágico (Levine et al., 2011).

O mecanismo da autofagia envolve vesículas de membrana dupla chamadas de autofagossomos ou vacúolos autofágicos, na qual as proteínas beclin-1 e proteínas de cadeia leve (LC3) são indispensáveis para a ocorrência de autofagia, pois participam na formação do autofagossomo (Levine & Yuan, 2005; Shintani & Klionsky, 2004). A LC3 ajuda a sequestrar as proteínas citosólicas e organelas para dentro do autofagossomo (Lamark, 2009) e a beclin-1 promove o recrutamento de membranas para formar o autofagossomo (Yue et al., 2003). De acordo com os autores, a LC3 é considerada como principal marcador da formação do autofagossomo e, desta forma sua detecção é uma forma confiável para se monitorar a autofagia (Tanida et al., 2008; Klionsky et al., 2012). Portanto, a autofagia é caracterizada pela conversão de LC3B-I em LC3B-II e pela expressão de beclin-1. O autofagossomo funde-se aos lisossomos para formar o autolisossomo, de membrana única, facilitando o processo de degradação e regeneração metabólica. Este processo pode assim, prolongar a sobrevivência celular durante a falta de nutrientes (Kelekar, 2006; Choi & Ryter, 2011).

Está descrito na literatura, que a autofagia está presente no trofoblasto extraviloso, sendo importante no início da gestação para promover a invasão trofoblástica e remodelação vascular uterina (Curtis et al., 2013; Hung et al., 2012; Nakashima et al.,

2013). Portanto, a ausência de autofagia no trofoblasto extraviloso no início da gestação, pode dificultar o processo de placentação. Outra possível função da autofagia é regular a resposta imune. Yuk et al. (2013) e Latz et al. (2013) descreveram que a autofagia regula negativamente o inflamassoma e subsequentemente a produção de IL-1β. Além disso, Shi et al. (2012) reportaram que a indução da autofagia depende da presença de sensores específicos do inflamassoma como o NLRP3. Assim, autofagia e inflamassoma aparentam ser mutualmente regulados.

Estudos sobre autofagia em placenta são escassos, sendo a maioria dos trabalhos desenvolvidos empregando-se linhagens celulares ou culturas de células trofoblásticas para avaliação *in vitro* de autofagia induzida por privação de nutrientes e de oxigênio (Curtis et al., 2013). De acordo com Oh et al. (2008), a expressão de LC3-II está aumentada em placenta de pacientes com pré-eclâmpsia grave, enquanto que a expressão de beclin-1 não apresentou diferenças significativas quando comparadas ao grupo controle. Além disso, Hung et al. (2012) observaram aumento na expressão de LC3-II em placenta de gestantes com PE, porém estas estavam acompanhadas de restrição de crescimento intra-uterino. No entanto, Goldman-Wohl et al. (2013) não encontraram diferenças significativas na expressão de genes relacionados à autofagia, comparando placentas de gestantes com pré-eclâmpsia e normotensas. Em trabalho realizado por Nakashima et al. (2013), através de estudos *in vitro*, observou-se que o fator anti-angiogênico, sEng, inibe a autofagia em células de trofoblasto extraviloso humano cultivadas sob condições de hipóxia (2%), reduzindo a expressão de LC3-II. Além disso, os autores observaram deficiência no processo autofágico em células de trofoblasto extraviloso humano obtidas de biópsia placentária de gestantes com PE. Assim, os resultados presentes na literatura são controversos e, desta forma é necessário compreender melhor o envolvimento da autofagia em patologias da gestação.

Segundo Bildirici et al. (2012) algumas questões devem ser levantadas para a compreensão desse fenômeno, como por exemplo: Qual é o papel da autofagia na homeostase do trofoblasto? ou ainda, a autofagia contribui para a disfunção placentária em patologias da gestação? Portanto, estudos sobre a regulação da autofagia em placenta poderão trazer novas informações sobre a função placentária sob condições de homeostase na gestação normal e na disfunção placentária em patologias da gestação.

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Objetívos

3. Objetivos

3.1. Objetivo geral

Investigar as proteínas relacionadas ao inflamassoma e autofagia em placenta de gestantes portadoras de pré-eclâmpsia e normotensas, bem como a relação existente entre inflamassoma e autofagia em células de trofoblasto extraviloso humano induzidas por anticorpo anti-fosfolípide.

3.2. Objetivos específicos

I. Avaliar a expressão de proteínas relacionadas ao inflamassoma e resposta inflamatória, NLRP3, caspase-1, IL-1β, TNF-α, IL-18 e HMGB 1 em placenta de gestantes portadoras de pré-eclâmpsia e de normotensas.

II. Avaliar a expressão de proteínas relacionadas à autofagia, LC3B-II, beclin-1 e mTOR, bem como avaliar a presença de autofagossomos por microscopia eletrônica de transmissão.

III. Investigar a relação existente entre inflamassoma e autofaiga em cultura de células de trofoblasto extraviloso humano de primeiro trimestre, induzidas por anticorpos anti-fosfolípides.

Resultados e discussão

4. Resultados e discussão

Os resultados e discussão dos dados obtidos encontram-se apresentados em forma de artigos científicos.

4.1. Artigo científico I

Inflammasome activation in placentas from pregnant women with preeclampsia (artigo submetido ao Journal of Reproductive Immunology)

4.2. Artigo científico II

Impaired placental autophagy in pregnant women with preeclampsia (manuscrito em preparação)

4.3. Artigo científico III

Relationship between autophagy and inflammasome activity in the human trophoblast aPL-induced (manuscrito em preparação)

Artigo científico I

Inflammasome activation in placentas from pregnant women with preeclampsia

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Abstract

Preeclampsia (PE) is a pregnancy disorder characterized by an imbalance between pro- and anti-inflammatory cytokines associated with high plasma levels of uric acid and Interleukin-1 beta (IL-1β) in severe forms of PE. The inflammasome is a protein complex that mediates innate immune responses via caspase-1 activation promoting secretion of IL-1β and IL-18 in their active forms, and also the release of the high-mobility group box 1 protein (HMGB1). As the placenta seems to play an important role in the pathogenesis of PE, the present study investigated the expression of genes and proteins related to the inflammasome in placentas from preeclamptic women. Placental tissue was collected from 20 normotensive pregnant women and 20 preeclamptic women, and inflammasome components, NLRP3 (NOD-like receptor family, pyrin domain-containing protein 3), caspase-1, IL-1β and IL-18, as well as tumor necrosis factor-alpha (TNF-α) and HMGB1 were evaluated by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) and also quantified by RT-qPCR. Compared with normotensive pregnant women, placenta from women with PE showed a significant increase in NLRP3, caspase-1, IL-1β, TNF-α and HMGB1 mRNA. Immunohistochemical staining of NLRP3, caspase-1, IL-1β and TNF-α in placental villi, as well as the levels of caspase-1, IL-1β, TNF-α and HMGB1 in placental homogenate were significantly higher in the preeclamptic group than in the normotensive group. However, mRNA expression of IL-18 and its protein concentrations were lower in placentas from preeclamptic women. The results suggest that placentas from pregnant women with preeclampsia show activation of NLRP3 inflammasome, which may be involved in the exaggerated inflammatory state in preeclampsia.

Keywords: inflammasome, NLRP3, IL-1β, placenta, preeclampsia

1. Introduction

Preeclampsia (PE) is an important cause of perinatal morbidity and mortality that affects between 2% and 8% of pregnancies (Duley, 2003; Kane et al., 2014) and is responsible for high maternal mortality in developing countries (Ghulmiyyah & Sibai, 2012). It is a multisystemic disorder with a clinical diagnosis based on the development of hypertension (BP ≥140 x 90 mmHg) and proteinuria (≥300 mg/24 h) that occurs from the 20th week of pregnancy on (NHBPEP, 2000; De Oliveira et al., 2010). Although the etiology of PE is not well understood, it is thought to be primarily associated with poor placentation, excessive inflammation and endothelial dysfunction (Redman & Sargent, 2005; Cheng & Sharma, 2016).

Abnormal placentation is considered to be the primary cause of placental hypoxia/ischemia, which leads to the release of numerous factors into the maternal circulation generating oxidative stress, a local inflammatory response and anti-angiogenic factor production. These products activate several pathways that contribute to clinical manifestations and disease progression (George & Granger, 2011; Warrington et al., 2013). Thus, the placenta seems to play a fundamental role in the development of PE.

In preeclamptic patients, excessive production of proinflammatory cytokines (Luppi & Deloia, 2006; Peraçoli et al., 2007; Raghupathy, 2013), deficient production of the regulatory cytokine Interleukin-10 (IL-10) in the placenta (Makris et al., 2006; Weel et al., 2016) and lower concentrations of IL-10 in the circulation or produced endogenously by monocytes from peripheral blood (Peraçoli et al., 2013; Cristofalo et al., 2013) have been reported. Also, a strong association between plasma levels of uric acid and IL-1β has been reported in severe cases of PE (Peracoli et al., 2013), suggesting that high levels of uric acid in the plasma of patients with PE may be related to the pathogenesis of PE by promoting the inflammatory response (Bainbridge & Roberts, 2008). Furthermore, Mulla

et al. (2011) demonstrated that uric acid acts as a danger-associated molecular pattern (DAMP) and activates the inflammasome in the trophoblast cells, leading to IL-1β secretion.

The inflammasome is a protein complex that mediates innate immune response via caspase-1 activation (Guo et al., 2015), which promotes the secretion of the pro-inflammatory cytokines IL-1β and IL-18, and also the release of the high-mobility group box 1 protein (HMGB1) (Schroder & Tschopp, 2010; Gross et al., 2011). The inflammasome is initially activated when the pattern-recognition receptors (PRRs), such as nucleotide oligomerization domain (NOD)-like receptors (NLRs), recognize the pathogen-associated molecular patterns (PAMPs) or endogenous products of host cells, also named danger-associated molecular patterns (DAMPs) (Guo et al., 2015). Oligomerization of nucleotide-binding domain and leucine-rich repeat pyrin domain containing protein-3 (NLRP3) inflammasomes bind to the inactive precursor form the caspase-1 enzyme, which becomes active and cleaves pro-IL-1β, resulting in biologically active IL-1β generation that is subsequently secreted into the extracellular medium (Pétrilli et al., 2007; Franchi et al., 2009).

High-mobility group box 1 protein (HMGB1) was originally described as a nuclear protein present in almost all cell types (Yang et al., 2013). When HMGB1 is released from cells into the extracellular environment following necrotic cell death, it acts as a proinflammatory mediator (Chen et al., 2016) and may contribute to the development of inflammatory responses in PE (Zhu et al., 2015). It has been suggested that HMGB1 may be involved in the pathogenesis of PE due to its higher expression in syncytiotrophoblast and in maternal serum from women with severe PE (Zhu et al., 2015).

There is scant available information on the role of inflammasomes in PE. A potential involvement of the inflammasome in PE is hypothesized as metabolites such as

acid uric and reactive oxygen species (ROS) could be considered danger signals driving inflammation (Khan & Hay, 2015). In a recent study, we demonstrated that mRNA expression of NLRP3, caspase-1, IL-1β and TNF-α by monocytes stimulated or unstimulated with monosodium urate (MSU) was significantly higher in preeclamptic women than in normotensive pregnant women. Also, monocytes from women with PE produced higher endogenous levels of IL-1β and TNF-α compared with the normotensive group, suggesting that uric acid plays a role in NLRP3 inflammasome activation contributing to the pathogenesis of PE (Matias et al., 2015).

Considering that the placenta seems to play an important role in the pathogenesis of PE, this study intended to investigate if there is activation of the inflammasome in the placentas of pregnant women with PE by evaluating gene and protein expression of NLRP3, caspase-1, IL-1β and IL-18, as well as TNF-α and HMGB 1 in placental tissue.

2. Materials and Methods

2.1. Subjects

Placentas were collected from 40 women with singleton pregnancies who delivered by elective cesarean section at the Obstetric Unit of Botucatu Medical School, Botucatu, SP, Brazil between March 2013 and December 2014. Twenty placentas were obtained from normotensive (NT) healthy pregnant women (controls) without hypertension-related complications who delivered at term (≥37 weeks gestation), and 20 placentas were collected from preeclamptic women with gestational age from 25 to 39 weeks. Placentas were collected by elective cesarean section, in the absence of delivery labor, for normotensive and preeclamptic groups. Gestational age of the groups was calculated from the last menstrual period and confirmed by early (<12 weeks gestation) ultrasound examination. Preeclampsia was defined as blood pressure ≥140/90 mmHg evaluated on

two occasions 2 h apart after 20 weeks of gestation and proteinuria of ≥300 mg/24 h in women with no previous history of hypertension (ACOG, 2002). Proteinuria in 24-h urine was measured by the Technicon RAXT automation system, and uric acid was assessed by uric acid enzymatic Trinder (Biotrol Diagnostic, Bridgewater, NJ, USA) in the Clinical Laboratory of Botucatu Medical School, Botucatu, SP, Brazil. Exclusion criteria included patients in labor, premature rupture of membranes, illicit drug use, and preexisting medical conditions such as diabetes, chronic hypertension and renal disease. The study was approved by the Ethics Committee of the Botucatu Medical School, and written informed consent was obtained from all women involved in the study. (CAAE Protocol number 349847). For pregnant women below 18 years of age, written informed consent was obtained from their parents or guardians.

2.2. Sample collection and preparation

Placentas were examined macroscopically according to previous guidelines (Kaplan et al.,1991) immediately after delivery. The tissues were obtained by cutting a vertical plane through the full thickness including the fetal and maternal surfaces. Tissues with calcification or clots were avoided. Samples of approximately 2 g of placental tissue were taken and used for immunohistochemical analysis, enzyme-linked immunosorbent assay (ELISA) quantification and quantitative real time polymerase chain reaction (RT-qPCR) evaluation.

2.3. Immunohistochemical analysis of placental tissues

Protein expression of NLRP3, caspase-1, IL-1β, IL-18, TNF-α and HMGB-1 was evaluated. Placental tissues embedded in paraffin were sectioned into 4-μm-thick slices and placed on histologic slides pretreated with Vectabond (Vector Laboratories Inc.,

Burlingame, CA, USA). Deparaffinization, rehydration and antigen recovery of the material was achieved using Trilogy buffer (Cell Marque Co, Rocklin, CA, USA) in a pressure cooker (Cell Marque) for 15 min. Then, sections were washed with phosphate buffered saline (PBS, pH 7.2), and treated with Peroxide Block (Cell Marque) for 10 min to block endogenous peroxidase and washed by successive baths in distilled water and PBS. The background was blocked using Background Block (Cell Marque) for 10 min followed by washes in distilled water and PBS. After blocking, sections were incubated for 60 min at 37 °C with antigen-specific primary anti-human antibodies. The concentrations of the antibodies were previously standardized using normal and preeclamptic placentas. The following antibodies with respective dilutions were used: rabbit polyclonal anti-NLRP3 (1/250), anti-IL-1β (1/50), anti-caspase 1 (1/50) (Novus Biologicals, Littleton, CO, USA), anti-IL-18 (1/100), anti-TNF-α (1/60) and anti-HMGB-1 (1/250) (Abcam Inc., Cambridge, MA, USA) diluted in antibody diluent (Cell Marque). After incubation, sections were washed in PBS and subjected to the action of signal amplifier for rabbit antibodies (Cell Marque) for 10 min at 37 °C. A polymer detector for rabbit antibodies (Cell Marque) was used for the detection of primary antibody with incubation for 10 min at 37 °C. After this step, sections were washed in PBS and incubated for 5 min in revealing solution containing 10 mg diaminobenzidine (DAB), 0.2% hydrogen peroxide (H_2O_2), 20 mM Trizma base and 1N HCl (Sigma). Sections were counterstained with Harris hematoxylin (Cell Marque) for 20 s, then bathed in running water. Dehydration was performed in sequential absolute ethanol baths of 90%, 80% and 70% alcohol, cleared in xylene (four baths) and mounted with coverslips containing Permount (Fisher Scientific, Fair Lawn, NJ, USA). To conduct the negative control reaction, primary antibody was replaced with a rabbit serum negative control (Cell Marque) containing the immunoglobulin isotype similar to the primary antibody used.

The expression of cytokines was identified in placental sections by an optical microscope (Olympus CX-31) with 10X ocular and 10, 20 and 40X objectives. Five random fields were photographed in every section of placenta with a 20X objective, and were analyzed by employing the software Image J. The quantification of the protein analyzed was obtained in pixels/μm/area.

2.4. Preparation of placental homogenates

To prepare placental homogenates, placental tissues were washed five times in ice-cold PBS to remove remaining blood. After this, 2 g of tissue was placed into a homogenate plastic tube containing 10 ml of ice-cold PBS and protease inhibitors (complete protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO, USA). The tissue was fully homogenized with a Powergen homogenizer (Fisher Scientific, Pittsburg, PA, USA) for 30 s on ice. Homogenates were centrifuged at 12,000 *g* for 20 min at 4 °C. The supernatant was collected, filtered through a 0.22 μm Millipore membrane, aliquoted and stored at –80 °C until required for cytokine (IL-1β, IL-18, TNF-α and HMGB1) and caspase-1 determination.

2.5. ELISA quantification

Concentrations of caspase 1, IL-1β, IL-18, TNF-α and HMGB1 in placental homogenates were determined by enzyme-linked immunosorbent assay (ELISA). Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) were employed for human caspase-1, IL-1β and TNF-α determination according to the manufacturer's instructions. Assay sensitivity limits were 1.24 pg/ml for caspase-1, 1.0 pg/ml for IL-1β and 5.5 pg/ml for TNF-α. Interleukin-18 was evaluated by a quantitative ELISA test kit for human IL-18 (MBL-Medical & Biological Laboratories, Nagoya, Japan) with a sensitivity

of 12.5 pg/ml, and HMGB1 was determined by an HMGB1 ELISA Kit (IBL Internacional – Shino Test, Hamburg, Germany), with a sensitivity of 0.1 ng/ml. Results were expressed as pg or ng/g of placental tissue.

2.6. Evaluation of the expression of transcripts related to inflammation

The placentas from normotensive and preeclamptic women were analyzed by expression of the genes encoding the proteins NLRP3, caspase-1, IL-1β, IL-18, TNF-α and HMGB-1 at the transcriptional level. Total RNA was extracted from the placentas using the Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) according to the manufacturer's protocol, and the Reverse Transcription-coupled polymerase chain reaction (RT-qPCR) was performed as described previously (Matias et al., 2015). Briefly, isolated RNA was DNAse I Amp Grade (Invitrogen) treated. Subsequently, the synthesis of complementary DNA (cDNA) was conducted using ImProm-IITM Reverse Transcription System, according to manufacturer's protocol. The RT-qPCR was made using RT GoTaq-qPCR Master Mix (Promega, Madison, WI, USA) and the primer sequences used in this study are listed in Table 1. Each reaction was set in duplicate and the conditions for the RT-qPCR were as follows: initial denaturation at 96°C for 2 min and then 40 cycles at 95°C for 15 s and 60°C for 60 s, followed by a melting curve. Expression values of the analyzed transcripts were normalized to that of the enzyme-encoding glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). The calculation of the differential expression of selected genes was carried out by the data processing method compared with a standard curve (Larionov et al.,2005). To analyze relative gene expression, we standardized the RNA expression levels in all samples to that of a single RNA sample, which was set to a value of 100.

Table 1

Primers for NLRP3, Caspase-1, IL-1β, IL-18, TNF-α, HMGB1 and GAPDH.

Gene	Forward primer	Reverse primer	GenBank
NLRP3	(2826)GAGGAAAAGGAAGGCCGACA(2845)	(2917)TGGCTGTTCACCAATCCATGA(2897)	NM_004895.4
Caspase1	(1065)AGACATCCCACAATGGGCTC(1084)	(1172)TGAAAATCGAACCTTGCGGAAA(1151)	NM_033292.3
IL-1β	(544)GAGCAACAAGTGGTGTTCTCC(564)	(653)AACACGCAGGACAGGTACAG(634)	NM_000576.2
IL-18	(438)ACTGTAGAGATAATGCACCCCG(459)	(517)AGTTACAGCCATACCTCTAGGC(496)	NM_001562.3
TNF-α	(325)GCTGCACTTTGGAGTGATCG(344)	(462)GGGTTTGCTACAACATGGGC(443)	NM_000594.3
HMGB1	(1404)TACGAAAAGGATATTGCTGC(1423)	(1505)CTCCTCTTCCTTCTTTTTCTTG(1484)	NM_001313893.1
GAPDH	(684)CGTGGAAGGACTCATGACCA(703)	(801)GGCAGGGATGATGTTCTGGA(782)	NM_002046.4

2.7. Statistical analysis

Statistical analyses for age, gestational age, blood pressure, proteinuria and uric acid, as well as gene expression and protein concentrations, were performed employing the Mann-Whitney *U* nonparametric test using the Prism software package. Differences between the groups studied were considered statistically significant when the *P* value was <0.05.

3. Results

3.1. Subject characteristics

Subject characteristics of all cases enrolled in this study are shown in Table 2. Maternal age was similar in the two groups evaluated. The gestational age at delivery was significantly lower in women with PE compared with NT pregnant women. Blood pressure values, proteinuria and uric acid levels were significantly higher while placental weight was significantly lower in the preeclamptic group than in the NT group.

Table 2
Clinical characteristics of the study population.

Characteristics	Normotensive (n = 20)	Preeclampsia (n = 20)
Age (years)	27 (17 -37)	27.5 (15 – 41)
Gestational age (weeks)	39 (37 – 40)	31 (25 – 39) *
Systolic blood pressure (mmHg)	110 (103 - 120)	165 (140 – 220) *
Diastolic blood pressure (mmHg)	80 (63 - 80)	110 (95 – 120) *
Proteinuria (mg/24 h)	< 300	3,120 (320 – 22,520) *
Uric acid (mg/dl)	3.9 (2.2 – 4.7)	6.6 (4.3 – 10.1) *
Placental weight (g)	502 (335 – 618)	270.5 (200 – 625) *

Results are expressed as medians and ranges.
* ($p < 0.05$) *vs* normotensive (Mann-Whitney *U* test)

3.2. Immunohistochemical analysis of NLRP3, caspase-1, IL-1β, IL-18, TNF-α and HMGB1 in placental tissues

According to our analysis, NLRP3, caspase-1, IL-1β, TNF-α, HMGB1 and IL-18 proteins were expressed by syncytiotrophoblast cells, mesenchymal cells and also by endothelial cells of the fetal capillaries as shown in Fig. 1 and Fig. 2.

All proteins evaluated showed the same localization in the two groups studied. However, differences in terms of the intensity of expression were found, and were quantified using the software Image J. The intensity of NLRP3, caspase-1 and IL-1β expression was significantly higher in the placentas of preeclamptic patients than in placentas of the NT group (Fig. 1 I-K). On the other hand, the expression of IL-18 was significantly lower in the placentas of preeclamptic patients compared with those of NT pregnant women (Fig. 1 L). Figure 2 shows that the expression of TNF-α was more intense in placental tissue of preeclamptic women than in NT pregnant women (Fig. 2 E), while no

significant differences were detected between the groups in relation to HMGB1 protein expression (Fig. 2 F).

3.3. Caspase-1, IL-1β, IL-18, TNF-α and HMGB1 detected in placental homogenate

Proteins related to inflammasome and to inflammatory responses were detected in placental homogenate by ELISA. Concentrations of caspase-1, IL-1β, TNF-α and HMGB1 were significantly higher in the preeclamptic group when compared with the NT group (Fig. 3A, 3B, 3D and 3E). However, the levels of IL-18 in placental homogenate were lower in preeclamptic patients than in the NT pregnant women (Fig. 3C).

3.4. Expression of genes related to inflammation in placental tissue

Expression of NLRP3, caspase-1, IL-1β, TNF-α and HMGB1 mRNA was significantly higher in placental tissue from pregnant women with PE compared with the normotensive pregnant group as shown in Fig. 4A, 4B, 4C, 4E and 4F respectively. On the contrary, lower gene expression of IL-18 was detected in placentas from the preeclamptic group than in those from the NT group (Fig. 4D).

4. Discussion

The results of the present study showed endogenous NLRP3 inflammasome activation in placenta from preeclamptic women demonstrated by higher expression of mRNA for NLRP3, caspase-1 and IL-1β, and their protein expression in placental villi detected by immunohistochemical analysis. Increased levels of caspase-1, IL-1β, TNF-α and HMGB1 were also detected in the placental homogenates of women with PE relative to those of NT pregnant women.

Inflammasome studies in the placenta are scarce. To our knowledge, this is the first report on inflammasome hyperactivity in placental tissue from pregnant women with PE. The involvement of NLRP3 inflammasomes in placental immune defense was shown by Pontillo et al. (2013). Stimulation of placental cell populations, such as human first trimester cytotrophoblasts and decidual stromal cells, with lipopolysaccharide (LPS), a common gram-negative compound, led to an augmented expression of caspase-1 and IL-1β, and the specific upregulation of NLRP3. Previous studies employing cultures of human first trimester villous trophoblasts and two trophoblast cell lines (Sw.71 and HTR-8/SVneo) showed that monosodium urate acts as DAMP, promoting IL-1β secretion via NLRP3-inflammasome activation, indicative of its proinflammatory effects. Inflammasome activation in trophoblast cells could be a mechanism of inflammation induction at the maternal-fetal interface that causes adverse pregnancy outcomes, including preeclampsia (Mulla et al., 2011).

Higher expression of TNF-α and IL-1β in placental villi and increased levels of these cytokines in placental homogenates from preeclamptic women demonstrated in the present study are in accordance with previous studies suggesting that ischemia and hypoxia, resulting from the inappropriate trophoblast invasion, lead to overexpression and increased production of proinflammatory cytokines in placentas from pregnant women with PE (Hung et al., 2004; Raghupathy, 2013). An association between higher levels of TNF-α and NLRP3 activation was detected in peripheral blood monocytes from preeclamptic women, both stimulated and unstimulated with monosodium urate (Matias et al., 2015), suggesting involvement of this inflammatory cytokine in inflammasome induction. A recent *in vitro* study demonstrated that the stimulation of a lineage of 3T3-LI mouse adipocytes with TNF-α induced mRNA expression of NLRP3 detected as early as

one hour after stimulation, suggesting that the Nlrp3 gene is an immediate gene responsive to TNF−α in these cells (Furuoka et al., 2016).

The molecular mechanism involved in placental NLRP3 activation in PE is unknown. It has been proposed that the excessive or inappropriate inflammatory response observed in PE may have its origin in the placenta. A potential mechanism in PE is the shedding of syncytiotrophoblast micro-vesicles and nano-vesicles from the placenta into the maternal circulation exerting proinflammatory, antiendothelial and procoagulant activity *in vitro*, all of which are features of the maternal syndrome (Sargent et al., 2003; Chen et al., 2016). It is conceivable that these microparticles can act as DAMPs that induce hyperactive inflammasome activity resulting in the exaggerated inflammatory state in PE (Khan & Hay, 2015). DAMPs are released from injured tissues and stressed or necrotic cells, and include degraded extracellular matrix components, heat shock proteins, HMGB1 protein and nucleic acids, among others. In PE, many such DAMPs are known to contribute to both local placental inflammation and to systemic inflammation and endothelial dysfunction. As in PE syncytiotrophoblast is subjected to oxidative and inflammatory stress, inflammatory molecules such as Hsp70, HMGB1, Galectin 3 and Synctin 1 are carried by the microvesicles (Iversen, 2013) and may act as DAMPs both in the placenta and peripheral blood mononuclear cells in patients with PE.

HMGB1 has been shown to be involved in the pathogenesis of several inflammatory diseases by acting as a critical mediator during infection and sterile injury (Andersson & Tracey, 2011; Yang et al., 2013). The present study showed significantly higher expression of mRNA and elevated levels of HMGB1 in placental tissues from preeclamptic women compared with those from the NT group. These results are in line with other recent studies in the literature showing increased mRNA levels of HMGB1 in placentas from women with severe PE, and its higher protein levels in maternal serum

(Zhu et al., 2015). Chen et al. (2016) reported that protein expression of HMGB1 was significantly increased in syncytiotrophoblast from placenta of both severe and early-onset PE compared with normotensive pregnancies. Although the precise role of HMGB1 in the placentas of women with PE remains to be elucidated, our results suggest that the NLRP3 inflammasome might mediate HMGB1 release from placental cells in response to the endogenous danger signals present in the damage tissues of placenta from preeclamptic women contributing to the inflammatory state detected in PE.

In the present study, IL-18 mRNA and cytokine expression in placenta were significantly lower in pregnant women with PE than in the NT control group. Previous studies reported lower levels of IL-18 in the serum of patients with PE (Sakai et al., 2004; Laskowska et al., 2011). This might be explained by the fact that IL-18 also stimulates the secretion of Th2 cytokines with anti-inflammatory activity (Klimkiewicz-Blok et al., 2013). Thus, the higher mRNA for IL-18 and its protein expression detected in placenta from the NT pregnant group compared with preeclamptic patients may contribute to the high IL-18 production to generate a protective Th2 immune response during pregnancy.

5. Conclusion

Together, our results demonstrated that placentas from pregnant women with PE show activation of the NLRP3 inflammasome and caspase-1 with higher expression of IL-1β, TNF-α and HMGB1. These results suggest that this inflammatory complex is involved in the pathogenesis of preeclampsia. The factors involved in placental inflammasome activation and the mechanisms for its control are the subject of future studies in our laboratory.

Conflicts of interest

The authors state that they have no financial or commercial conflicts of interest.

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Figure legends

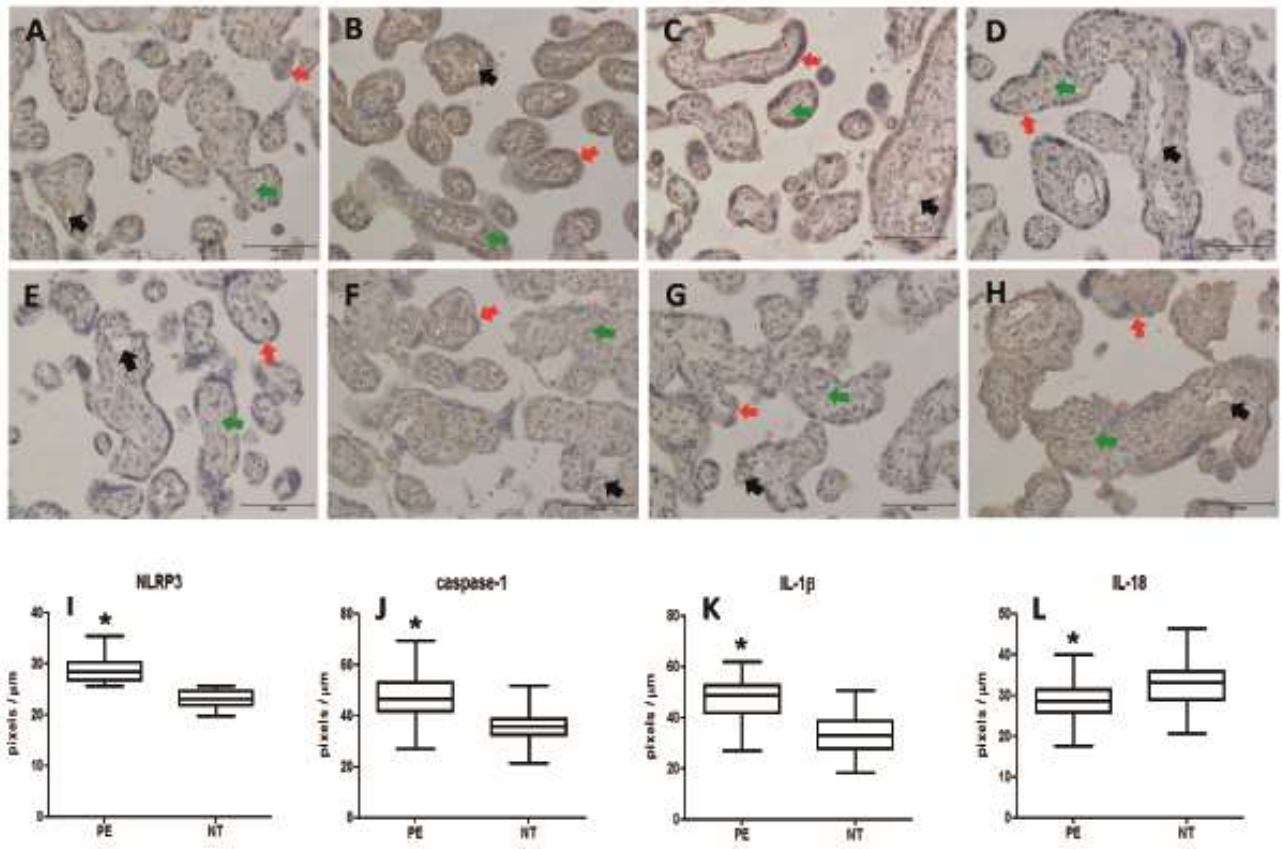
Fig. 1. Immunohistochemical staining for NLP3, caspase-1, IL-1β and IL-18 in placenta from women with preeclampsia (PE) (A–D) and from normotensive (NT) pregnant women (E-H). Red arrow shows positivity in the syncytiotrophoblast, green arrow shows positivity in the cytoplasm of mesenchymal cells and black arrow shows positivity in the fetal capillary endothelium.
Quantitative analysis of NLRP3 (I), caspase-1 (J), IL-1β (K) and IL-18 (L) expression by placental tissues from women with preeclampsia (PE) and from normotensive (NT) pregnant women. Results are represented in pixels/μm. *($p < 0.05$) *vs* normotensive group.

Fig. 2. Immunohistochemical staining for TNF-α and HMGB1 in placentas from women with preeclampsia (PE) (A, B) and from normotensive (NT) pregnant women (C, D). Red arrow shows positivity in the syncytiotrophoblast, green arrow shows positivity in the cytoplasm of mesenchymal cells and black arrow shows positivity in the fetal capillary endothelium.
Quantitative analysis of TNF-α (E) and HMGB1 (F) expression by placental tissues from women with preeclampsia (PE) and from normotensive (NT) pregnant women. Results are represented in pixels/μm. * ($p < 0.05$) *vs* normotensive group.

Fig. 3. Concentrations of caspase-1 (A), IL-1β (B), IL-18 (C), TNF-α (D) and HMGB1 (E) in placental homogenates from women with preeclampsia (PE) and from normotensive (NT) pregnant women. Results are represented in pg/ml or ng/ml *($p < 0.05$) *vs* normotensive group.

Fig. 4. Gene expression of NLRP3 (A), caspase-1 (B), IL-1β (C), IL-18 (D) TNF-α (E) and HMGB1 (F) in placental tissues from women with preeclampsia (PE) and from normotensive (NT) pregnant women. Results are shown as median. *($p < 0.05$) *vs* normotensive group.

Figure 1


A
B
C
D
E
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G
H
NLRP3
I
pixels / µm
PE
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caspase-1
J
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NT
IL-18
L
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NT

Figure 2

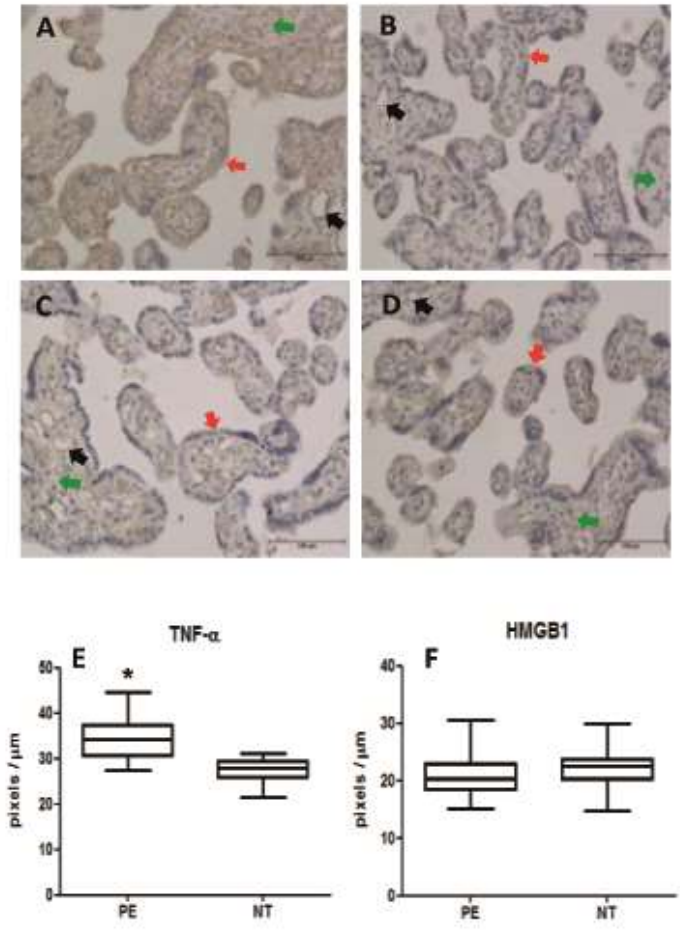
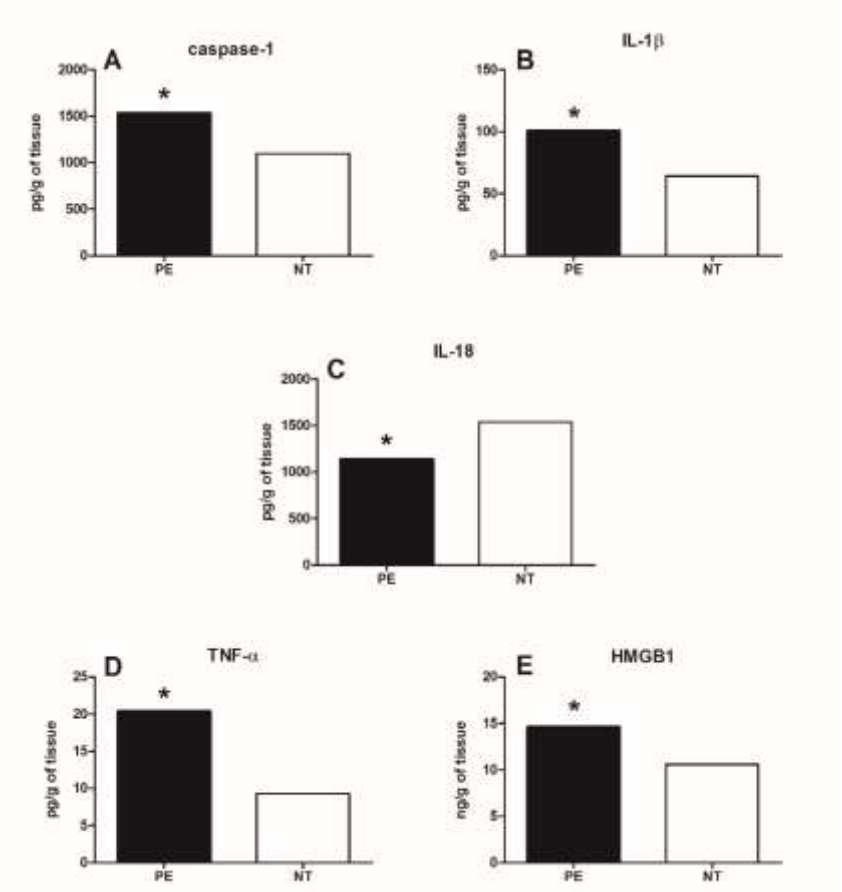

A
B
C
D
TNF-α
E
*
pixels / µm
0
10
20
30
40
50
PE
NT
HMGB1
F
pixels / µm
0
10
20
30
40
PE
NT

Figure 3


A
caspase-1
pg/g of tissue
2000
1500
1000
500
0
*
PE
NT
B
IL-1β
pg/g of tissue
150
100
50
0
*
PE
NT
C
IL-18
pg/g of tissue
2000
1500
1000
500
0
*
PE
NT
D
TNF-α
pg/g of tissue
25
20
15
10
5
0
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PE
NT
E
HMGB1
ng/g of tissue
20
15
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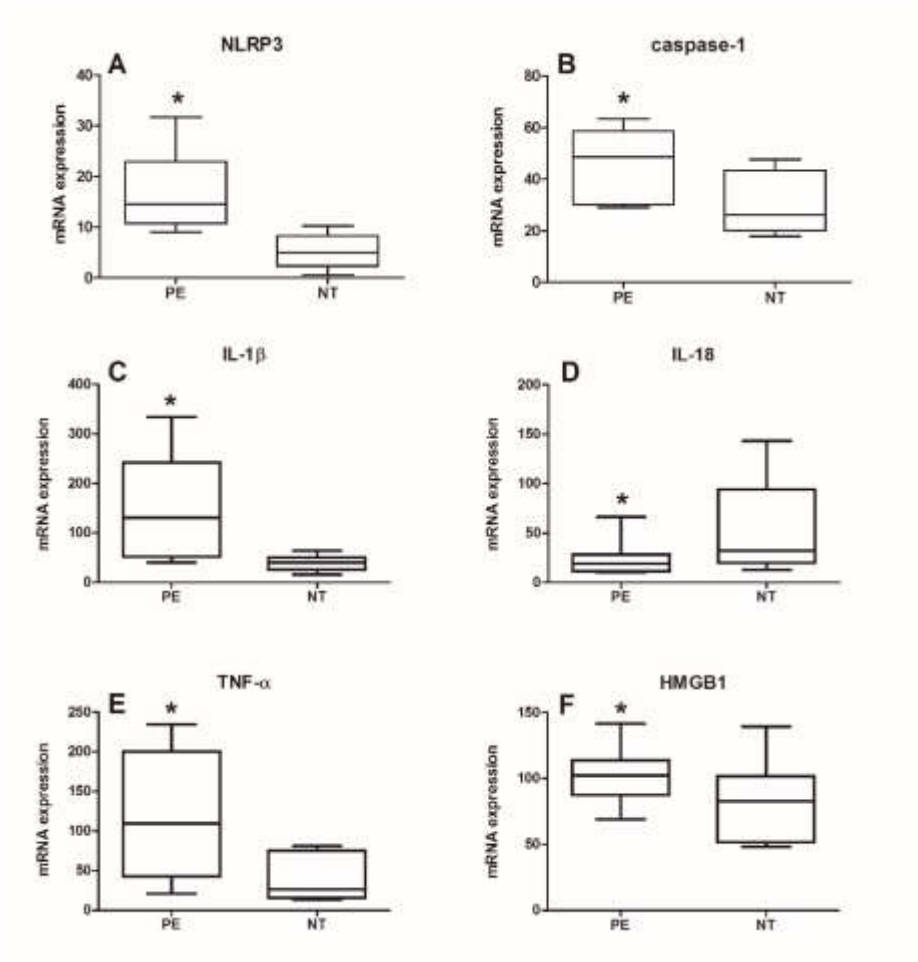
0
*
PE
NT

Figure 4


NLRP3
A
mRNA expression
PE
NT
caspase-1
B
IL-1β
C
IL-18
D
TNF-α
E
HMGB1
F

Artigo científico II

Impaired placental autophagy in pregnant women with preeclampsia

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Abstract

Autophagy is a lysosomal degradation pathway that removes protein aggregates and damaged organelles maintaining the cellular integrity. It seems to be essential for cell survival during stress, starvation, hypoxia and consequently to the placenta implantation and development. Preeclampsia (PE) is a multisystemic disorder with the clinical diagnosis based on the onset of hypertension and proteinuria after the 20^{th} week of gestation. Considering that placenta seems to play an important role in the pathogenesis of PE the objective of the present study was to evaluate in placenta of pregnant women with PE the proteins related to the autophagy such as light chain protein (LC3B-II), beclin-1 and the mammalian target of rapamycin (mTOR). Placental tissues collected from 20 women with PE and from 20 normotensive pregnant women were employed for LC3B-II, beclin-1 and mTOR expression by immunohistochemistry and real-time RT-PCR. The presence of autophagosome was evaluated by electron microscopy. The mRNA for LC3B-II and beclin-1 and their protein expression were significantly decreased in placenta of pregnant women with PE than in normotensive pregnant women. On the other hand mTOR gene and protein expression were significantly increased in placenta from pregnant women with PE compared with normotensive pregnant women. There is a negative correlation between protein expression of mTOR and LC3B-II in placental tissue of preeclamptic women ($r = -0.3433$; $p = 0.0022$). In conclusion, we showed that placenta from preeclamptic patients had an autophagy impairment, suggesting that failure in this degradation process may contribute to the pathogenesis of PE.

Keywords: autophagy, beclin-1, LC3B-II, mTOR, placenta, preeclampsia

Introduction

Autophagy is a catabolic lysosomal degradation process that degrades cytoplasmic materials including proteins aggregates and damaged organelles [Czaja et al., 2013; Ni et al., 2014], maintaining the cellular homeostasis and promoting the cell survival during stress [Hung et al., 2013]. This autophagy process is dependent on the mammalian target of rapamycin (mTOR) inhibition by nutrient deprivation which is described to attenuate autophagy when it is increased [Levine et al., 2011].

During autophagy, double membrane vesicles called autophagosomes or autophagic vacuoles are responsible for sequestering proteins and damaged organelles. These autophagosomes are mainly formed by beclin-1 and light chain protein (LC3) which are necessary for the occurrence of autophagy [Shintani & Klionsky, 2004; Levine & Yuan, 2005]. The activation of the autophagy is represented by an increase in the concentration of these key proteins pathways [Klionski et al., 2012]. LC3 is a cytosolic protein present in mammal cells, which is converted to LC3-II and recruited to the autophagosome membranes during the autophagic process and is considered the principal marker associated with formation of the completed autophagosome. Thus, according to the authors, the LC3 detection is a reliable means to monitor autophagy [Tanida et al, 2008; Klionsky et al, 2012]. LC3 helps to sequester cytosolic proteins and organelles into the autophagosome [Lamarck, 2009] and beclin-1 promotes the recruitment of membranes to form the autophagosome [Yue et al., 2003]. Therefore, autophagy is characterized by autophagosomes with the conversion of LC3B-I to LC3B-II and beclin-1 expression. The autophagosomes fuses with lysosomes to form autolysosomes facilitating then, the process of metabolic degradation and regeneration. Thus, this phenomenon can prolong cell survival in the absence of nutrients [Kelekar, 2006; Choi & Ryter, 2011].

Studies of autophagy in the placenta are scarce. Most of the studies use cultures of cell lines or trophoblast cells to evaluate autophagy induced by nutrient deprivation and oxygen [Curtis et al., 2013]. Some studies reveal that autophagy is present in extravillous trophoblast cells and is important in early pregnancy to promote trophoblastic invasion and uterine vascular remodeling [Hung et al., 2012; Nakashima et al., 2013]. During normal placentation the maternal spiral arterioles are invaded by extravillous trophoblast cells, resulting in remodeling of these vessels to ensure an adequate blood supply to the placenta and fetus. However, in women with preeclampsia (PE) trophoblastic invasion is inadequate, causing defects in the spiral arteriole remodeling that results in poor perfusion [Brosens et al., 1972; George & Granger, 2011].

Preeclampsia is characterized by the new-onset hypertension and proteinuria that occurs from 20 weeks of pregnancy affecting between 2% and 8% of human pregnancies and constitutes the major cause of maternal and perinatal morbidity and mortality [NHBPEP, 2000; Kane et al., 2014]. Although the aetiology of PE are poorly understood, some studies suggest that the pathophysiology of this pregnancy disorder involves both maternal and placental factors [Redman & Sargent, 2005].

The abnormal placentation seems to be the primary cause of placental underperfusion/hypoxia/ischemia, leading to the release of factors into the maternal circulation which generate oxidative stress, inflammatory response and anti-angiogenic factors production. These factors may be account for the systemic inflammatory response and endothelial dysfunction [Redman & Sargent 2005, Warrington et al., 2013]. The preeclamptic patients are characterized to produce excessive proinflammatory cytokines [Redman & Sargent, 2003; Luppi & Deloia, 2006; Raghupathy 2013] as well as lower production of Interleukin-10 (IL-10), a cytokine with downregulatory activity [Orange et al., 2003; Cristofalo et al., 2013; Peraçoli et al., 2013]. Recently, we reported increased

levels of tumor necrosis factor-alpha (TNF-α) and fms-like tyrosine-kinase-1(Flt-1) associated with lower levels Interleukin 10 (IL-10), vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in placentas from women with early-onset PE versus normotensive placentas [Weel et al., 2016]. The proinflammatory and anti-angiogenic state detected suggest that placenta play a fundamental role in the pathogenesis of PE. Thus, the present study sought to investigate the proteins related to the autophagy in placental tissue from preeclamptic women by the evaluation of LC3B-II, beclin-1 and mTOR and also the occurrence of the autophagosome by electron microscopy.

Material and Methods

Subjects

Placentas were collected from 40 women with singleton pregnancies who delivered at the Obstetric Unit of Botucatu Medical School, Botucatu, SP, Brazil between March 2013 and December 2014, by elective cesarean section in all groups. Twenty placentas were collected from normotensive healthy pregnant women (controls) without hypertension-related complications that delivery at term (≥37 weeks gestation), and 20 placentas were collected from pregnant women with PE. Gestational age of the groups was calculated from the last menstrual period and confirmed by early (<12 weeks gestation) ultrasound examination. Preeclampsia was defined as blood pressure ≥140/90 mmHg evaluated on two occasions 2 h apart after 20 weeks of gestation and proteinuria of ≥300 mg/24 h in women with no previous history of hypertension [ACOG, 2002]. Proteinuria was measured by the Technicon RAXT automation system. Exclusion criteria included patients in labor, premature rupture of membranes, illicit drug use, and preexisting medical conditions such as diabetes, chronic hypertension and renal disease. The study was approved by the Ethics Committee of the Botucatu Medical School, and the written

informed consent was obtained from all women involved in the study (CAAE Protocol number 349847). For pregnant women with age below 18 years old the written informed consent was obtained from their parents or guardians.

Sample collection and preparation

Placentas were macroscopically examined according to previous guidelines [Kaplan et al., 1991], immediately after delivery. The tissues were obtained by cutting a vertical plane through the full thickness including the fetal and maternal surfaces. Samples of approximately 2 g of placental tissue were taken and used for immunohistochemical analysis, quantitative Polymerase Chain in real time (RT-qPCR) and electron microscopy evaluation.

Immunohistochemical analysis of placental tissues

The expression of LC3B-II, beclin-1 and mTOR were evaluated. Placental tissues embedded in paraffin were sectioned into 4 μm thick slices and placed on histologic slides, pretreated with Vectabond (Vector Laboratories Inc., Burlingame, CA, USA). Deparaffinization, rehydration and antigen recovery of the material was obtained using Trilogy buffer (Cell Marque Co, Rocklin, CA, USA) in a pressure cooker (Cell Marque) for 15 min. Then, sections were washed with phosphate buffered saline (PBS, pH 7.2), and treated with Peroxide Block (Cell Marque) for 10 min to block endogenous peroxidase and then washed by successive baths in distilled water and PBS. The background was blocked using Background Block (Cell Marque) for 10 min followed by washes in distilled water and PBS. After blocking, sections were incubated for 60 min at 37 °C with antigen-specific primary anti-human antibodies. The concentrations of the antibodies were previously standardized using normal and preeclamptic placentas. The following antibodies with

respective dilutions were used: rabbit policlonal anti-LC3B-II [1/300], anti-beclin-1 [1/100] and anti-mTOR [1/100] (Novus Biologicals, Littleton, CO, USA) diluted in Antibody Diluent(Cell Marque). After incubation, sections were washed in PBS and subjected to the action of signal Amplifier for rabbit antibodies (Cell Marque) for 10 min at 37 °C. A Polymer Detector for rabbit antibodies (Cell Marque) was used for detection of primary antibody with incubation for 10 min at 37 °C. After this step sections were washed in PBS and incubated for 5 min in revealing solution containing 10 mg of diaminobenzidine (DAB), 0.2% of hydrogen peroxide (H2O2) and Trizma base -20mm, 1N HCl (Sigma). Sections were counterstained with Harris hematoxylin (Cell Marque) for 20 sec, and then bathed in running water. Dehydration was performed in absolute ethanol sequential baths, alcohol 90%, 80% and 70%, cleared in xylene (four baths) and mounted with coverslips containing Permount(Fisher Scientific, Fair Lawn, NJ, USA).

To conduct the negative control reaction, primary antibody was replaced with a rabbit serum negative control (Cell Marque) containing the immunoglobulin isotype similar to the primary antibody used.

The expression of autophagy proteins was identified in placental sections by an optical microscope (Olympus CX-31) with 10X ocular and 10, 20 and 40X objectives. Five random fields were photographed in every section of placenta with a 20x objective, and were analyzed by employing the software Image J. The quantification of the protein analyzed was obtained in pixels/μm/area.

Expression of transcripts related to autophagy

Placentas from normotensive and preeclamptic women were subjected to analysis of the expression, of the gene encoding the proteins LC3B-II, beclin-1 and mTOR at the transcriptional level, Total RNA was extracted from the placentas through the system Total

RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) according to manufacturer's protocol. After extraction, to ensure complete removal of genomic DNA, 1 μg of total RNA was incubated with DNAse I Amp Grade (Invitrogen). The purity and relative quality of all samples of total RNA obtained were determined by fluorometry using the equipment Qubit Fluorometric Quantitation (Life Technologies). Subsequently, the synthesis of complementary DNA (cDNA) for performing the polymerase chain reaction coupled reverse transcription (Reverse Transcription-coupled polymerase chain reaction—RT-PCR) was conducted using ImProm- IITM Reverse Transcription System, according to manufacturer's protocol. The quantification of gene expression of LC3B-II, beclin-1 and mTOR was made through the reaction technique in quantitative Polymerase Chain in real time (RT-qPCR) using RT GoTaq-qPCR Master Mix (Promega, Madison, WI, USA). The device used was 7500 Fast Real Time PCR Systems (Applied Biosystems, USA). The variants of the targets studied were aligned in the MEGA 5.1 program and subsequently each primer was selected by the software Primer-BLAST. Primers located in exon-exon junction guarantee the purity of the reaction, namely the absence of any genomic DNA that may contaminate it. The primer sequences used in this study are in Table 1. Each reaction was set in duplicate in a total of 20μL each, which contains 0,3μM of each primer (forward and reverse), 2 μL of cDNA, 10 μL of master mix and 6 μL of nuclease-free water. Additionally, was inserted a control, also in duplicate, which was included in each reaction in order to prove that there is no contamination. The conditions for the RT-qPCR reactions were: initial denaturation at 96°C—2 min and then 40 cycles at 95°C- 15s and 60°C -60s, followed by a melting curve. The amplification of each particular transcript was confirmed by melting curve generated profile of the end of each reaction. Expression values of the analyzed transcripts were normalized based on the concurrent analysis of the expression of the enzyme encoding glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). The

calculation of the differential expression of selected genes was carried out by the data processing method compared to a standard curve [Larionov et al., 2005]. To analyze the relative expression, after the analysis of gene expression, we chose an RNA sample obtained, which received the relative value of 100. All other samples received values for that sample.

Table 1.Primers for LC3B-II, beclin-1, mTOR and GAPDH.

Gene	Forward primer	Reverse primer	GeneBank
LC3B-II	(517)CCAGGAAACCTTCGGCTTCT(536)	(632) CGGTAGAGGCAGCTCAGTTC (613)	NM_032514.3
beclin-1	(28) TCGCTGAAGACAGAGCGATG (37)	(151) CGATGCTCTTCACCTCGGG (133)	NM_003766.3
mTOR	(4170)TCGCTGAAGTCACACAGACC(4189)	(4307) CTTTGGCATATGCTCGGCAC (4288)	NM_004958.3
GAPDH	(684)CGTGGAAGGACTCATGACCA(703)	(801)GGCAGGGATGATGTTCTGGA(782)	NM_002046.4

Electron microscopy

Placental tissues were fixed in 2.5% glutaraldehyde for 12 hours, flushed in 0.1M phosphate buffer at pH 7.3, fixed in a 1% osmium tetroxide solution for 1 hour, flushed in phosphate buffer, dehydrated in increasing solutions of 75% to 100% alcohol, and dried in a critical point device (Balzers CPD-020; Balzers AG, Balzers, Liechtenstein) with liquid carbon dioxide. The fragments were assembled in a metal base with silver glue and then covered with gold (15 nm gold) in a Balzers MED-010 device. They were visualized and photographed at increasing magnitudes under a scanning electron microscope (Quanta 200 FEG; FEI Company, Munich, Germany).

Statistical analysis

Statistical analyses for age, gestational age, blood pressure, proteinuria, as well as LC3B-II, Beclin-1 and mTOR proteins concentrations, were performed employing the Mann-Whitney *U* nonparametric test. The correlation coefficient (r) was determined by using Spearman rank correlation test. All statistical analyses were performed using Prisma Statistical software 9 version 5.0 (GraphPad San Diego, Calif., U.S.A.). Differences were considered statistically significant at $P < 0.05$.

Results

Subject characteristics

Subject characteristics of preeclamptic and normotensive pregnant women are shown in Table 2. Maternal age was similar in the two groups studied. The gestational age at delivery was significantly lower in women with PE compared with NT pregnant women. Systolic and diastolic blood pressure and proteinuria values were significantly higher in PE group than in NT group.

Table 2. Subject characteristics

Characteristics	Normotensive	Preeclampsia
	(n=20)	(n=20)
Age (years)	27 (18 – 36)	28 (16 – 41)
Gestational age (weeks)	38 (37 – 40)	31 * (26 – 39)
Systolic blood pressure (mmHg)	115 (103 - 120)	165 * (140 – 210)
Diastolic blood pressure (mmHg)	70 (65 - 80)	112 * (98 – 120)
Proteinuria (mg/24 hours)	< 300	3,750 * (320 – 19,750)

The values are expressed as median and range.
* ($p<0.05$) *vs* normotensive (Mann-Whitney *U* test)

Immunohistochemical analysis of LC3B-II, beclin-1 and mTOR in placental tissues

The proteins LC3B-II, beclin-1 and mTOR were expressed by syncytiotrophoblast cells, mesenchymal cells and also expressed by endothelial cells of the fetal capillaries as shown in figure 1. All proteins evaluated showed the same localization in the two groups studied. However, differences in intensity of expression were found, and were quantified using the software Image J (Fig 1 G-I). The intensity of LC3B-II and beclin-1 expression was significantly lower in placentas of preeclamptic patients than in placentas of the normotensive group (Fig 1 G-H). However, mTOR expression was significantly higher in placenta of pregnant women with PE when compared to normotensive pregnant women (Fig 1I). Association analysis between mTOR and LC3B-II in placental tissue showed

negative correlation in preeclamptic women (r = - 0.3433; p = 0.0022). No significant correlation between these parameters (r = - 0.1905; p = 0.1142) was observed in the NT pregnant group. Also, there was no significant correlation between mTOR and beclin-1 in preeclamptic (r = - 0.2050; p = 0.0819) and in normotensive (r = - 0.1612; p = 0.1825) groups.

Expression of genes related to autophagy in placental tissue

Figure 2A and 2B show, respectively, lower gene expression of LC3B-II and beclin-1 in placental tissue from pregnant women with PE when compared to NT. On the other hand, gene expression of mTOR was significantly higher in placental tissue from pregnant women with PE than in the normotensive group (Fig 2C).

Autophagy analysis by electron microscopy in placental tissue

In normal pregnant women (Fig 3A-C) and in women with PE (Fig 3D-G), syncytiotrophoblast cells showed an irregularly form, with numerous extensions covered by plasma membrane (Fig 3A and 3D), similar to microvilli. The cytoplasm of these cells in NT pregnant women displayed flocculated content vacuoles with low density (Fig 3B). There were also some small mitochondria, rare multivesicular bodies (Fig 3B) and some dense content autophagosomes (Fig 3B and 3C). The nucleus was in an irregular form containing decondensed chromatin and few heterochromatic clumps attached to the nuclear envelope (Fig 3B).

In pregnant women with PE the cytoplasmic ultrastructure of the cells was different from normal pregnant group. It was possible to observe a greater number of heterogeneous content vacuoles in the cytoplasm (Fig 3E), some myelin figures (Fig 3G), few mitochondria (Fig 3D), multivesicular bodies (Fig 3F) and autophagosomes with a larger

size and different levels of intracellular digestion contents (Fig 3F and 3G). The nucleus was irregular with higher amounts of heterochromatin clumps attached to the nuclear envelope (Fig 3D).

Discussion

The results of the present study show that there is deficiency in the autophagy phenomenon in placenta of pregnant women with PE. Lower detection of mRNA for LC3B-II and beclin-1 was associated with decreased expression of these proteins in syncytiotrophoblast of placental tissue from pregnant women with PE compared with NT pregnant women. On the other hand, mRNA for mTOR and its expression was significantly increased in placenta of preeclamptic women. In order to verify the autophagosome presence, we analyzed placental tissue by transmission electronic microscopy, and observed some dense content inside autophagosomes in placental tissue of NT pregnant women and few autophagosomes with a larger size and different levels of intracellular digestion contents in placenta of pregnant women with PE.

The study of autophagy in human placenta is recent, and the results in the literature are controversial. Autophagy related to pregnancies complicated by PE was first reported by Oh et al. [2008] followed by others [Hung et al., 2012; Akaishi et al, 2014]. Other studies described association of autophagy with normal pregnancies resolved by vaginal delivery or by cesarean section, or with gestational pathologies such as intrauterine growth restriction [Signorelli et al, 2011; Chang et al, 2013;. Curtis et al., 2013; Avagliano et al, 2013; 2013a].

Our results showing autophagy deficiency in placenta from pregnant women with PE are in disagreement with some reports of the literature above cited. According to Oh et al. [2008], LC3-II expression is increased in placenta from severe PE compared with

control pregnant women, while beclin-1 shows no difference between the groups. On the other hand, LC3-II overexpression was not detected in PE alone, but was observed only in the placenta of preeclamptic pregnant women when associated with fetal growth restriction [Hung et al., 2012]. Autophagy activation was demonstrated by increase in LC3-II and decrease in p62 proteins examined by western blot in placenta of preeclamptic women even in the absence of intrauterine growth restriction [Akaishi et al., 2014]. Furthermore, Gao et al. [2015] quantified LC3, beclin-1 and autophagosome in placental tissue by immunohistochemistry, western blot and RT-PCR, and found a significantly increased expression of LC3 and beclin-1 in placentas from pregnancies complicated by early-onset preeclampsia. According to the authors the excessive autophagic activity may be involved in the development of PE. However, Goldman-Wohl et al. [2013] showed no significant differences in autophagy associated gene expression in preeclamptic versus normal placental samples. These reports show that the occurrence of autophagy in placenta of pregnant women with PE deserves better studies.

The lower autophagic activity in placenta of pregnant women with PE, detected in this study, could be related to systemic and placental inflammatory process observed in this pathology. Although the pathogenesis of PE has not yet been fully determined because the complexity and variability of this disorder, it seems to originate in the placenta [Redman & Sargent, 2009] leading to phenomena associated with this syndrome, such as excessive inflammatory response [Redman & Sargent, 2010], premature aging of the placenta, hypoxia and placental insufficiency, characteristics associated with impaired autophagy (Goldman-Wohl et al., 2013). The placental and endothelial lesions resultant from these phenomena lead to the release of anti-angiogenic factors such as the soluble form of vascular endothelial growth factor receptor vascular (VEGFR-1 or sFlt-1) and soluble endoglin (sEng), a co-receptor for factor transforming growth and beta (TGF-β1), which

induce inflammatory responses and systemic endothelial dysfunction resulting in hypertension and proteinuria after 20 weeks of gestation (Levine et al., 2006). In vitro studies performed by Nakashima et al. [2013] showed that sEng inhibits autophagy in extravillous trophoblast cells cultured under hypoxic condition (2%), reducing LC3-II expression as detected by western blot. The authors also demonstrated deficiency of autophagy in extravillous trophoblast cells obtained from biopsies of placental bed of preeclamptic women. These results suggest that the impairment of autophagy induced by sEng, could play a role in systemic inflammation observed in PE [Saito & Nakashima, 2014].

In a recent study, we demonstrated an imbalance in cytokines and angiogenic factors, represented by higher TNF-α/IL-10 and sFlt-1/PlGF ratios in placental tissue associated with more severe lesions in women with early-onset PE [Weel et al., 2016]. Thus, the higher inflammatory and anti-angiogenic state observed in placenta from severe forms of PE could be involved in placental insufficiency and led to impaired autophagy pathways.

Our results showing negative correlation between decrease in expression of LC3B-II and higher expression of mTOR in placenta from preclamptic women suggest a compensator regulatory mechanism of autophagy played by mTOR activation in insufficient and inflamed placenta. In a recent study we reported that placenta from pregnant women with preeclampsia shows activation of NLRP3 inflammasome, demonstrated by significant increase of mRNA for NLRP3, caspase-1, IL-1β, TNF-α and HMGB1. Immunohistochemical staining of NLRP3, caspase-1, IL-1β and TNF-α in placental villi, as well as the levels of caspase-1, IL-1β, TNF-α and HMGB1 in placental homogenate were significantly higher in preeclamptic group than in normotensive group (Weel et al., 2016a). Increased expression of mTOR has been observed in inflammatory

diseases. Upregulation of mTOR gene expression in the peripheral blood mononuclear cells of patients with osteoarthritis is accompanied by increased synovial inflammation and might serve as an indicator of disease activity [Tchetina et al., 2013]. Ortiz-Masiá et al. [2014] demonstrate impaired autophagy in a murine model of colitis and in the intestinal mucosa of patients with inflammatory bowel diseases. Decreased expression of LC3II in colonic epithelial crypts from damaged mucosa was associated with increased protein levels of b-catenin and phosphorylated mTOR. According to the authors the activation of mTOR, a central negative regulator of autophagy, was detected in the damaged area, suggesting that colonic inflammation and injury may be associated with impaired autophagy. Considering these recent results of mTOR regulatory role on autophagy, the association between mTOR activation and inflammation needs to be better studied in PE.

The autophagy and inflammasome pathways are linked by mutual regulation. Autophagy controls inflammation through interactions with innate immune pathways, by removing endogenous inflammasome components, and affects the secretion of immune mediators [Abdelaziz et al., 2015]. Thus, autophagy induced by inflammatory signals targets ubiquitinated inflammasome, thereby limiting IL-1 β production through inflammasome destruction [Shi et al., 2012]. On the other hand, depletion of beclin-1 and LC3B in mouse macrophages upregulates caspase-1 activation and secretion of mature IL-1β and IL-18 in response to LPS in an NLRP3 inflammasome-dependent manner [Nakahira et. al.,2011]. Blockage of autophagy treatment of macrophage cells with 3-methyl adenine (3-MA) resulted in the production of mitochondrial reactive oxygen species (mtROS) and NLRP3-dependent IL-1β secretion in the absence of traditional inflammasome stimulants [Zhou et al., 2010]. This suggested that when damaged mitochondria were not cleared by autophagy, they released mtROS which stimulated the

NLRP3 inflammasomes with mature IL-1β secretion [Zhou et al., 2010; Rogers et al., 2014].

In conclusion, we showed that placenta of preeclamptic patients has an autophagy deficiency characterized by lower LC3B-II and beclin-1 expression and higher mTOR expression. This autophagy impairment may be involved in the excessive inflammatory response seen in the placenta of pregnant women with preeclampsia [Weel et al., 2016; 2016a]. Studies involving cross-talk between autophagy and inflammasome pathways in placenta from women with preeclampsia might help to understand the impairment of autophagy described in the present study.

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Figure legends

Fig. 1. Immunohistochemical staining for of LC3B-II, beclin-1 and mTOR in placenta from women with preeclampsia (PE) (A-C) and in normotensive (NT) pregnant women (D–F) respectively. Red arrow shows positivity in the syncytiotrophoblast, green arrow shows positivity in cytoplasm of mesenchymal cells and black arrow shows positivity in the fetal capillary endothelium.

Quantitative analysis of LC3B-II (G); beclin-1 (H) and mTOR (I) expression by placental tissues from normotensive(NT) pregnant women and women with preeclampsia (PE). Results are represented in pixels/μm. * ($p < 0.05$) *vs* normotensive group.

Fig 2. Gene expression of LC3B-II (A), beclin-1 (B) and mTOR (C) in placental tissues from pregnant women with preeclampsia (PE) and normotensive (NT) pregnant women. Results are represented as median values. * ($p < 0.05$) *vs* normotensive group.

Fig3. Ultraestructure of syncytiotrophoblast from placenta of normotensive pregnant women (A-C) and from women with preeclampsia (D-G). Syncytiotrophoblast cells presents an irregularly form, with numerous extensions covered by plasma membrane (A); nucleus with irregular form containing decondensed chromatin and few heterochromatic clumps, and cytoplasm with vacuoles, mitochondria, rare multivesicular bodies and autophagosomes (arrow head) (B); dense content autophagosome (arrow head) in evidence (C); Syncytiotrophoblast cells exibiting an irregularly form, with numerous extensions covered by plasma membrane, few mitochondria and irregular nucleus with higher amounts of heterochromatin clumps (D); greater number of vacuoles in the cytoplasm (E); presence of multivesicular bodies and autophagosome (arrow head) (F) myelin figures and autophagosomes (arrow head) with a larger size and different levels of intracellular digestion contents in the cytoplasm(G).

Abreviations- E: extensions, N: nucleus, Mi: mitochondria, MB: multivesicular bodies, V: vacuoles, MF: myelin figure.

Figure 1

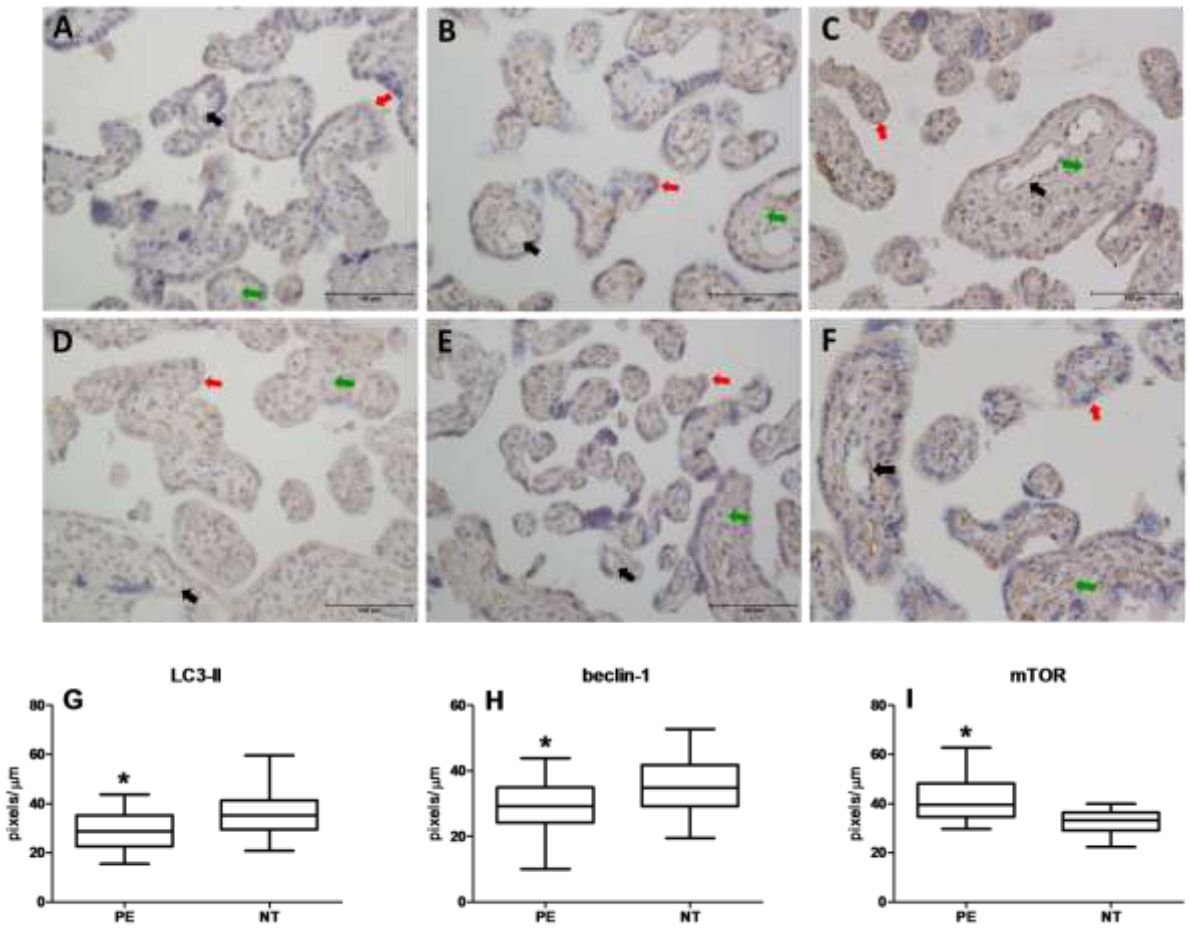
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Figure 2

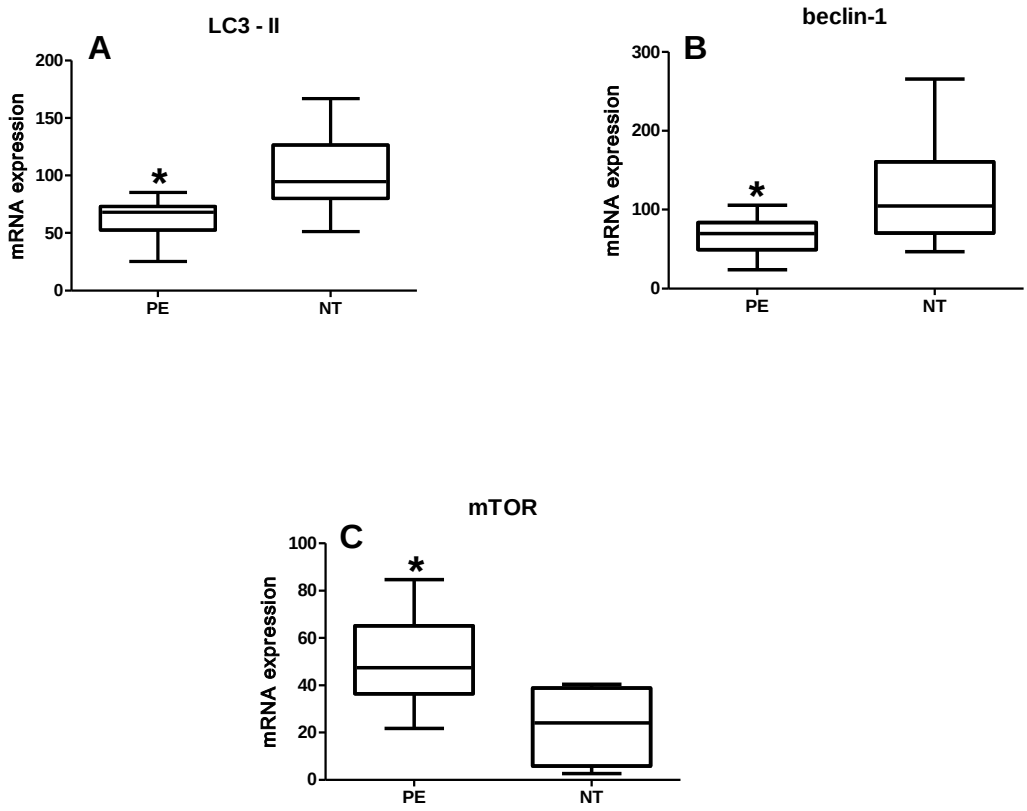
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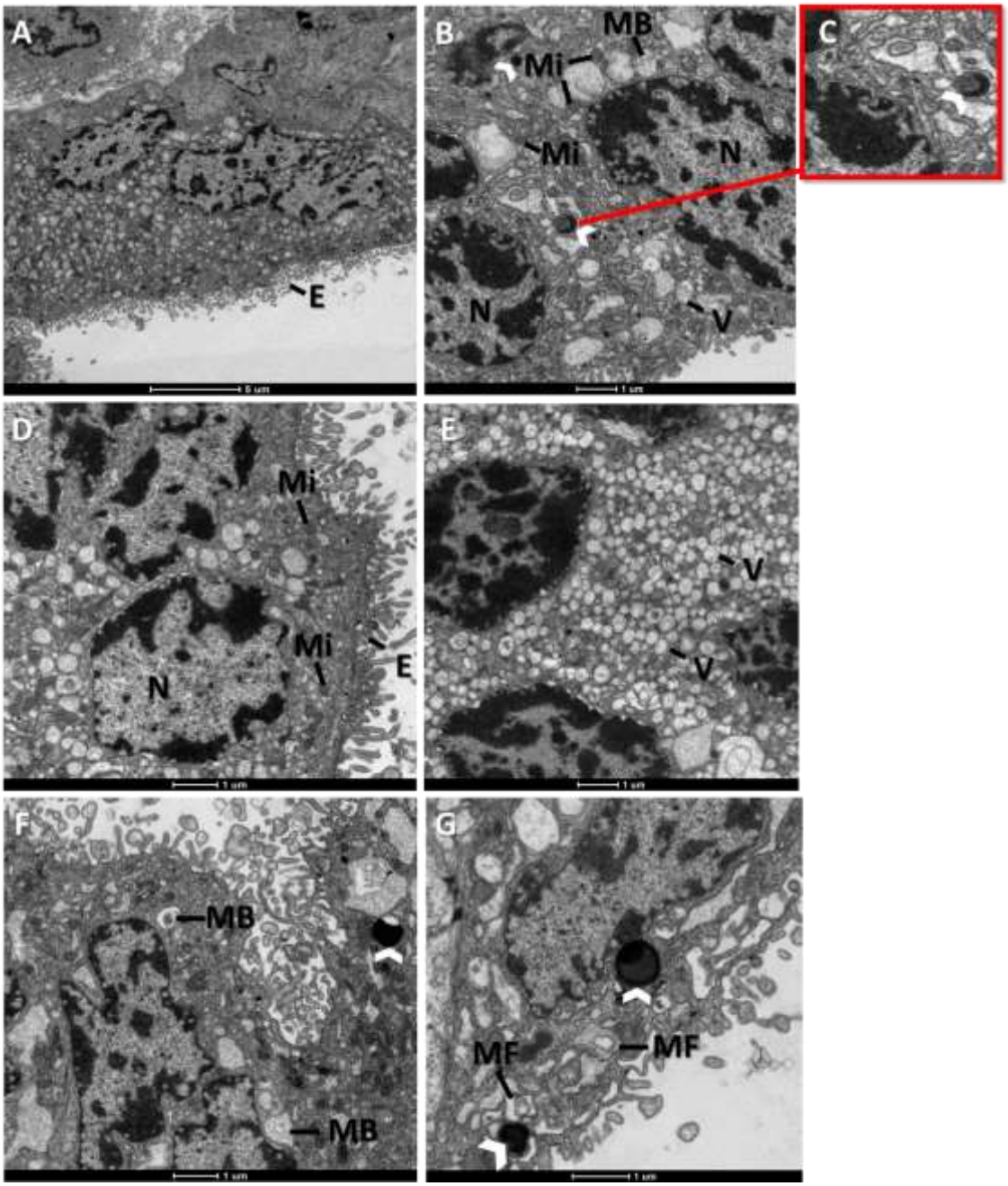
Figure 3



Artigo científico III

Relationship between autophagy and inflammasome activity in the human trophoblast aPL-induced

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Abstract

Introduction: Antiphospholipid syndrome (APS) is characterized by the presence of circulating antiphospholipid antibodies (aPLs). aPL are a family of autoantibodies that react with placental trophoblast expressing beta$_2$ glycoprotein I (β_2GPI), inducing human trophoblast inflammation, by triggering the cell to secrete elevated levels of pro-inflammatory cytokines, such as Interleukin 1 beta (IL-1β) and Interleukin 8 (IL-8). Autophagy is a regulatory process that facilitates the degradation and recycling of cytoplasmic components, but it can also regulate inflammatory responses. **Objectives:**Using aPL as a model of Nalp3 inflammasome activation and inflammation, we sought to investigate its crosstalk with autophagy in human first trimester extravillous trophoblast cells. **Methods:** A human first trimester extravillous trophoblast cell line, SW.71, was treated with or without mouse anti-human β2-glycoprotein I monoclonal antibodies (aPL). The autophagy response was determined by the evaluation of light chain protein (LC3BI/II), beclin-1 and p62 employing western blot analysis. Cells were treated with bafilomycin or rapamycin to inhibit or stimulate the autophagy process, respectively. IL-1β, IL-8, soluble engdoglin (sEng), soluble fms-like tyrosine-kinase-1 (sFlt-1) and uric acid secretion, by SW.71 cells, were measured by ELISA. **Results:** aPL significantly decreased the trophoblast LC3B-II/I ratio after 8hrs and significantly increased the LC3B-II/I ratio after 72hrs when compared to the controls. There were no differences in the expression levels of either beclin 1 or p62. Bafilomycin significantly increased trophoblast secretion of IL-1β, IL-8 and sEng. Rapamycin alone significantly decreased trophoblast sFlt-1 and sEng in comparison to the control. Rapamycin significantly reduced aPL-induced secretion of IL-1β and IL-8, but had no effect on aPL-induced uric acid, sEng or sFlt-1 production. **Conclusion**: The results showed that aPL impair trophoblast autophagy which is turn allows inflammasome activation and subsequent IL-1β production, as well as

Toll-like receptor 4 (TLR4)-mediated IL-8 secretion to occur. In addiction a later induction or recovery of trophoblast autophagy may be triggered by the inflammasome.

Keywords: antiphospholipid antibodies, autophagy, inflammasome and trophoblast.

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by an elevated risk of thrombosis and/or obstetrical complications (Costedoat-Chalumeau et al., 2012; Danza et al., 2012). It is diagnosed by the presence of persistent high titres of circulating antiphospholipid antibodies (aPLs), as well as repeated miscarriage (Hughes, 1983).

Antiphospholipid antibodies are a heterogeneous family of autoantibodies that react with phospholipid-binding proteins, such as beta$_2$ glycoprotein I (β_2GPI). This targeting is particularly pathologic in obstetric APS (Meroni et al., 2011; Meroni et al., 2012; Oku et al., 2012) since the antigen is constitutively expressed by the placental trophoblast; especially the extravillous trophoblast population that invades the decidua and remodels the uterine spiral arterioles (Chamley et al.,1997; Quenby et al., 2005). In APS patients, pregnancy complications are associated with inflammation at the maternal-fetal interface (Berman et al., 2005; Girardi et al., 2003; Mulla et al., 2009; Redecha et al.,2007; Van Horn et al 2004; Sebire et al., 2003) and with placental insufficiency associated with reduced trophoblast invasion and limited spiral artery remodeling (Alvarez et al.,2015; Mulla et al., 2010; Bose et al., 2006; Sebire et al., 2002).

It is demonstrated that aPL recognizes β_2GPI triggering human first trimester trophoblast cells to produce elevated pro-inflammatory Interleukin 1 beta (IL-1β) and Interleukin 8 (IL-8) levels (Mulla et al., 2009; Mulla et al., 2013) and increase trophoblast secretion of the anti-angiogenic factor, soluble endoglin (sEng), (Carroll et al., 2011).

Mulla et al. (2009) reported that the aPL-induced IL-1β secretion occurs after pro-IL-1β is processed into its active form. This process is commonly mediated by the Nalp3 inflammasome, a complex of Nalp3 that subsequently activates caspase-1 (Bergsbaken et al.,2009; Fink & Cookson, 2006; Silveira & Zamboni, 2010) and promotes the secretion of

the pro-inflammatory cytokine IL-1β (Schroder & Tschopp, 2010; Gross et al., 2011). The use of trophoblast cells where Nalp3 was knocked using small interfering RNA (siRNA), demonstrated that aPL-induced IL-1β secretion is indeed mediated by activation of the Nalp3 inflammasome through endogenous uric acid production (Mulla et al., 2013).

Autophagy is a regulatory process that facilitates the degradation and recycling of cytoplasmic components via lysosomes (Klionsky et al., 2000). During autophagy, proteins and organelles are sequestered into double-membrane vesicles called autophagosomes. Among these proteins, beclin-1 and light chain protein (LC3) are essential for the occurrence of autophagy, and play a role in the autophagosome formation (Levine & Yuan, 2005; Shintani & Klionsky, 2004). Autophagosomes fuse with lysosomes to generate single-membrane autophagolysosomes and the sequestered material is degraded and metabolized by the cell. These autophagic functions are important for cellular homeostasis and can promote cell survival in response to environmental stresses such as starvation and hypoxia (Curtis et al., 2013).

Autophagy is differentially regulated in different trophoblast populations on the placenta. In normal pregnancy, the villous syncytiotrophoblast normally lacks markers of autophagy. In contrast, basal autophagy occurs in the extravillous trophoblast population, the cells of interest in APS, and this may be important early in pregnancy for promoting trophoblast invasion and uterine vascular remodeling (Curtis et al., 2013; Hung et al., 2012; Nakashima et al., 2013).

Another possible function of autophagy is to regulate innate immune function and inflammation. Yuk et al (2013) and Latz et al (2013) reported that autophagy is a negative regulator of inflammasome activity and subsequent IL-1β production. Conversely, Shi et al. (2012) reported that the induction of autophagy depends on the presence of specific

inflammasome sensors, such as Nalp3, for selective degradation. Thus, autophagy and inflammasome activity appear to be co-dependent.

While increased inflammasome activity is associated with placental inflammation (Mulla et al., 2011 and 2013), nothing is known about the relationship between these two pathways in extravillous trophoblast cells. Using aPL as a model of Nalp3 inflammasome activation and inflammation, we sought to investigate its crosstalk with autophagy in human first trimester extravillous trophoblast cells. Inflammasome and autophagy mutual regulation was accesed by the evaluation of the aPL effect on human first trimester extravillous trophoblast cell autophagy and by determining the role of autophagy on trophoblast inflammatory and angiogenic responses to aPL by either inhibiting or stimulating autophagy.

Methods

Trophoblast Cells

The human first trimester extravillous trophoblast telomerase-immortilized cell line, Sw.71 (Straszewski-Chavez et al., 2009) was used for this study. Cells were cultured in DMEM (Gibco-Invitrogen; Grand Island, NY), supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10 mM Hepes, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate and 100 nM penicillin/streptomycin (Gibco-Invitrogen) and maintained at 37˚C/5% CO2. These cells exhibit characteristics of an extravillous trophoblast and function similarly to primary cultures (Straszewski-Chavez et al., 2009; Mulla et al., 2009; Mulla et al., 2013). To stimulate autophagy, trophoblast cells were treated with rapamycin [500nM] (Invivogen, CA, USA) and to inhibit autophagy, trophoblast cells were treated with bafilomycin [0.5μM] (Invivogen, CA, USA).

Treatment with antiphospholipid antibodies

The aPL, mouse IgG1 anti-human β_2GPI monoclonal antibody (IIC5 and ID2) were used in this study. Like human aPL, both antibodies have similar reactivity to human aPL and bind to β_2GPI (Chamley et al., 1999). These aPL also bind to first trimester trophoblast cells, and similarly to patient-derived polyclonal aPL, alter trophoblast function, inducing IL-1β production (Carroll et al.,2011; Mulla et al., 2009; Mulla 2013). Mouse IgG1, clone 107.3 (BD Pharmingen, San Jose) served as an isotype control. For all experiments, trophoblast cells were treated in serum-free OptiMEM with or without IIC5, ID2 or IgG (20μg/mL). After 8 and/or 72hrs, cell-free supernatants were collected and cells were lysed for protein.

Western Blot Analysis

For analysis of proteins associated with autophagy we performed Western blot. Protein lysates were diluted with gel loading buffer and boiled for 5 minutes, after which they were resolved under reducing conditions on 12% SDS-PAGE gels and then transferred onto PVDF membrane (PerkinElmer, Boston, MA). Membranes were blocked with 5% fat-free powdered milk (FFPM) in PBS/0.05% Tween-20 (PBS-T). Following washes with PBS-T, membranes were incubated overnight at 4˚C with the following primary antibodies diluted in PBS-T/1% FFPM: LC3B-I/LC3B-II [1:5000] (Rabbit Ab, Cell Signaling), beclin-1 [1:5000] (Rabbit Ab, Santa Cruz Biotech), p62 [1:2000] (mouse mAb, MBL). Following this incubation, membranes were washed with PBS-T and then incubated with a 1:10.000 dilution of either the goat anti-rabbit or horse anti-mouse IgG secondary antibody conjugated to peroxidase (Vector Labs; Burlingame, CA) in PBS-T/1% FFPM. Following washes with PBS-T and then with distilled water, the peroxidase-conjugated antibody was detected by enhanced chemiluminescence (PerkinElmer). hsp-90 (Rabbit Ab, Santa Cruz Biotech) was used as an internal loading control to validate the

amount of protein loaded onto the gels. Images were recorded and semi-quantitative densitometry was performed using the Gel Logic 100 and Carestream software (Eastman Kodak, Rochester, NY).

Measurement of IL-1β, IL-8, sEndoglin, sFlt-1 and Uric acid

The concentration of IL-1β, IL-8, sEng and soluble fms-like tyrosine-kinase-1 (sFlt-1) in culture supernatants were quantified by ELISA according to the manufacturer's instructions (R&D Systems; Enzo Life Science). For the measurement of uric acid, culture supernatants were analyzed according to the manufacturer's instructions using the QuantiChrom assay kit from BioAssay Systems (Hayward, CA).

Statistical Analysis

Experiments were performed at least three times and assayed in triplicate. Statistical significance set to $p<0.05$ was determined using Prism software (GraphPad Software, Inc, La Jolla, CA, USA). For multiple comparisons, significance was determined using one-way ANOVA followed by Bonferroni´s post-hoc test.

Results

Effect of aPL on human first trimester extravillous trophoblast cell autophagy

To evaluate the effect of aPL on trophoblast autophagy we have determined LC3B-I and LC3B-II at 8 and 72 hours. As shown in Figure 1A and B, after 8 hrs treatment of trophoblast cells with the aPL, ID2 and IIC5 significantly decreased the LC3BII/LC3BI ratio by 41.3% and 59.8% respectively, when compared to the non-treated (NT) control ($p<0.05$). The IgG isotype control had no effect on basal LC3BI or LC3BII expression levels. In contrast, after 72 hrs treatment of trophoblast cells with the aPL, ID2 and IIC5

significantly increases the LC3BII/LC3BI ratio by 35.2% and 14.5% respectively, when compared to the NT control (Figure 1A and C; $p<0.05$). Again at this time point, the IgG isotype control had no effect on basal LC3BI or II expression levels. Together this data indicates that there is a biphasic autophagic response to aPL; at the early time point aPL reduced basal trophoblast autophagy, while at the later time point this autophagy response was increased. Having established this differential LC3B response to aPL, two other markers of autophagy were evaluated; beclin 1 and p62. As shown in Figure 2, neither the aPL (ID2 or IIC5) nor the IgG control had any effect on trophoblast expression levels of beclin 1 or p62 at either time point. Thus, we focused our remaining studies on LC3B as our autophagy marker.

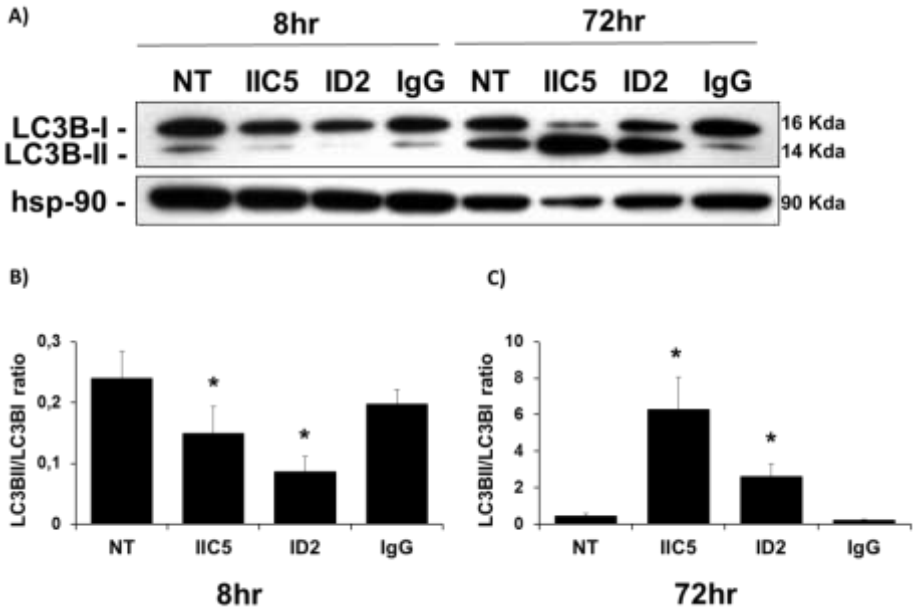

Fig 1. LC3B I and II expression in human trophoblast cells after treatment with aPL. Trophoblast cells were cultured with no treatment (NT), the aPL, ID2 or IIC5, or the IgG control for at 8hr and 72 hr. A) Representative western blot for LC3BI, LC3BII and the loading control, hsp-90 ;B&C) Barcharts show LC3BII/LC3BI ratio as determined by densitometry and normalized to hsp-90 (n=3). *$p<0.05$ *vs* to the NT control.

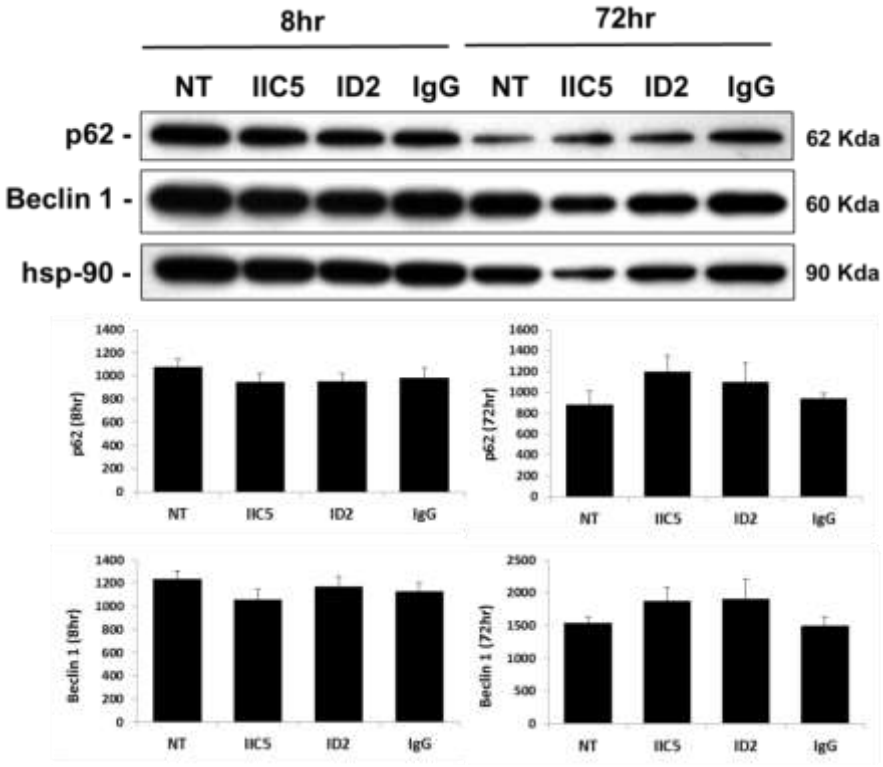

Fig 2. p62 and Beclin 1 expression in human trophoblast cells after treatment with aPL. Trophoblast cells were cultured with no treatment (NT), the aPL, ID2 or IIC5, or the IgG control for at 8hr and 72 hr. Representative western blot for p62, Beclin 1 and the loading control, hsp-90. Barcharts show p62 and Beclin-1 as determined by densitometry normalized to hsp-90 (n=3).

The role of autophagy in trophoblast inflammatory and angiogenic responses to aPL

To determine the impact of aPL-mediated inhibition on the subsequent inflammasome/inflammatory response as well as angiogenic factor production, trophoblast autophagy was either inhibited or stimulated. To mimic the aPL effect of autophagy trophoblast inhibition, bafilomycin was employed as an autophagy inhibitor. Conversely, in the presence of aPL, autophagy was maintained using rapamycin. After 72hr the measurements were made for uric acid, IL-1β and IL-8 secretion, as markers for inflammasome function and inflammation; sEng and sFlt-1, as markers of the angiogenic response.

As shown in Figure 3A (i), inhibition of autophagy using bafilomycin had no effect on the levels of uric acid secretion, an upstream trigger of the Nalp3 inflamamsome. Similarly, maintainance of autophagy using rapamycin has no effect on the ability of aPL to increase uric acid production as compared to the NT control (Figure 3A ii & iii). However, the IL-1β secretion was altered. As shown in Figure 3B (i), bafilomycin significantly increased IL-1β secretion (392.6±60) fold compared to the NT control. Conversely, aPL-induced IL-1β secretion was significantly inhibited by the presence of rapamycin (Figure 3B ii & iii). The other inflammatory marker, IL-8 was also regulated in a similar manner. As shown in Figure 3C (i), bafilomycin significantly increased IL-8 secretion 1.8±0.15 fold compared to the NT control. Conversely, aPL-induced IL-8 secretion was significantly inhibited by the presence of rapamycin (Figure 3C ii & iii).

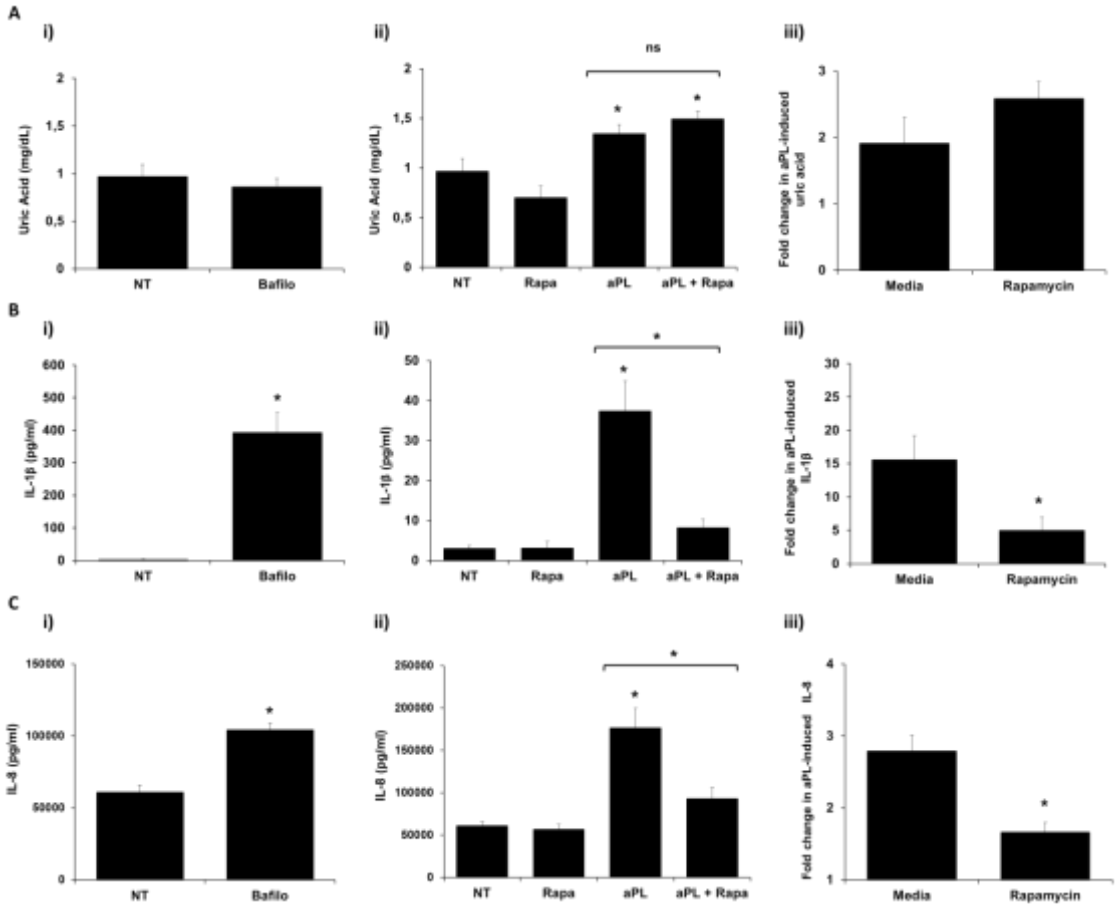

Fig 3. Trophoblast inflammasome and inflammatory response to autophagy inhibition or stimulation. A) Uric acid secretion 72hr after: i) either no treatment (NT) or treatment with bafolimycin (Bafilo); or ii) either no treatment (NT) or treatment with rapamycin (Rapa), IIC5 (aPL), or Rapa + aPL. iii) shows the data from ii) presented as aPL + Rapa/Rapa fold change relative to the aPL/NT (n=7). B) IL-1β secretion 72hr after: i) either no treatment (NT) or treatment with bafolimycin (Bafilo); or ii) either no treatment (NT) or treatment with rapamycin (Rapa), IIC5 (aPL), or Rapa + aPL. iii) shows the data from ii) presented as aPL + Rapa/Rapa fold change relative to the aPL/NT (n=7).C) IL-8 secretion 72hr after: i) either no treatment (NT) or treatment with bafolimycin (Bafilo); or ii) either no treatment (NT) or treatment with rapamycin (Rapa), IIC5 (aPL), or Rapa + aPL. iii) shows the data from ii) presented as aPL + Rapa/Rapa fold change relative to the aPL/NT (n=7). *$p<0.05$ relative to the NT control.

Inhibition of autophagy using bafilomycin significantly increased the secretion of sEng by 2±0.16 fold compared to the NT control (Figure 4A i), but had no effect on sFlt-1

secretion (Figure 4B i). When autophagy was maintained using rapamycin, the aPL-induced decreased significantly sEng (Figure 4A ii) and sFlt-1 (Figure 4B ii) secretion. Moreover, rapamycin alone also significantly reduced sEndoglin (Figure 4A ii) and sFlt-1 (Figure 4B ii) secretion when compared to the NT control but, there was no differences on the fold change with aPL + Rapa/Rapa relative to the aPL/NT, thus there was no true effect on aPL response (Figure 4A iii & 4B iii).

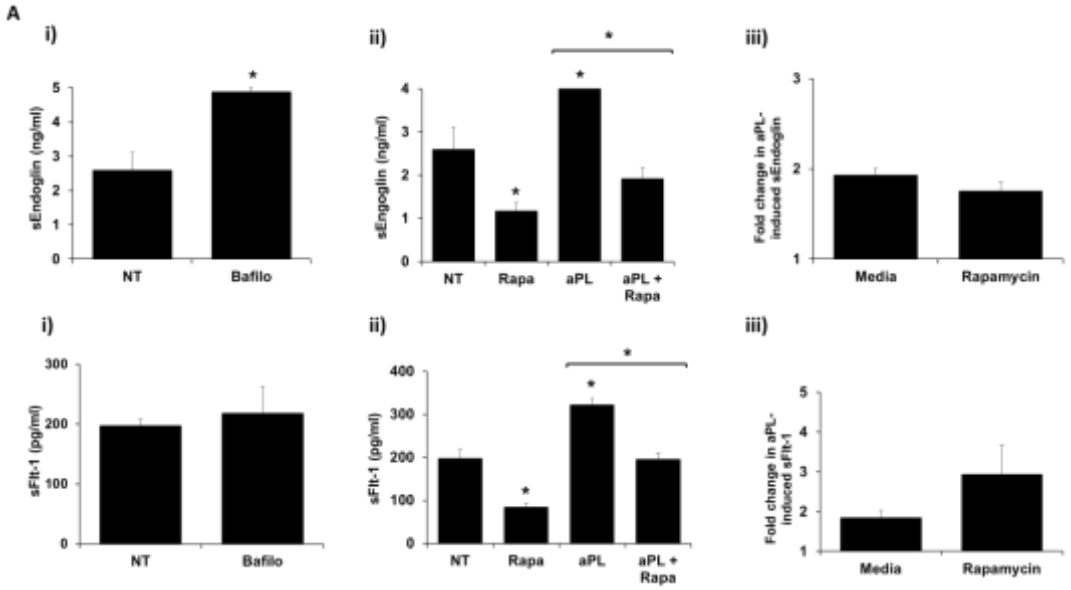

Fig 4. Trophoblast anti-angiogenic response to autophagy inhibition or stimulation. A) sEndoglin secretion 72hr after: i) either no treatment (NT) or treatment with bafolimycin (Bafilo); or ii) either no treatment (NT) or treatment with rapamycin (Rapa), IIC5 (aPL), or Rapa + aPL. iii) shows the data from ii) presented as aPL + Rapa/Rapa old change relative to the aPL/NT (n=7). B) sFlt-1 secretion 72hr after: i) either no treatment (NT) or treatment with bafolimycin (Bafilo); or ii) either no treatment (NT) or treatment with rapamycin (Rapa), IIC5 (aPL), or Rapa + aPL. iii) shows the data from ii) presented as aPL + Rapa/Rapa fold change relative to the aPL/NT (n=7). *$p<0.05$ relative to the NT control.

Discussion

Antiphospholipid antibodies are known to induce human trophoblast inflammation, triggering the cell to secrete elevated levels of the pro-inflammatory cytokines IL-1β and

IL-8 (Mulla et al.,2009). Furthermore, it is known that aPL-induced IL-1β secretion is mediated by activation of the Nalp3 inflammasome through endogenous uric acid production (Mulla et al., 2013). It has also been reported that autophagy is an inhibitor of the inflammasome and that there may be bi-directional regulation between autophagy and inflammasome responses (Shi et al., 2012; Latz et al., 2013; Yuk et al., 2013;) and Thus this study sought to examine this relationship in the trophoblast in the setting of aPL.

The results of the present study showed a lower LC3BII/I ratio 8hr after exposure to aPL while at the later time point of 72hr aPL increased the LC3BII/I ratio. This suggests that aPL trigger a bi-phasic autophagy response in the trophoblast by reducing this process early and inducing it later. We hypothesized that aPL-mediate impaired autophagy allowing subsequent inflammasome activity and inflammation to occur, but this the autophagy process was recovered later, potentially due to the its activation by inflammasome activation. The autophagy and inflammasome are linked by mutual regulation. Autophagy controls inflammation through interactions with innate immune pathways, by removing endogenous inflammasome components, and affects the secretion of immune mediators (Abdelaziz et al., 2015). Thus, autophagy induced by inflammatory signals sequester inflammasome, thereby limiting IL-1 β production through inflammasome destruction (Shi et al., 2012).

Indeed, in this study we found that inhibiting the basal trophoblast autophagic process with bafilomycin, in the absence of aPL, lead to the IL-1β secretion decrease similarly to aPL effect. Furthermore, the aPL-induced trophoblast IL-1β response could be blocked when autophagy was maintained by rapamycin. This finding confirmed that the trophoblast autophagy negatively regulates Nalp3 inflammasome function. Moreover, this inhibition appears to be at the level of the inflammasome since the upstream trigger of

aPL-induced Nalp3 activation and uric acid was not regulated by bafilomcyin or rapamycin.

It is known that aPL-induced trophoblast IL-8 secretion is not mediated by the Nalp3 inflammasome but it is mediated by Toll-like receptor 4 (TLR4) (Mulla et al.,2009; Mulla et al., 2013) and our results showed that the aPL-mediated impairment of autophagy also negatively regulates the IL-8 secretion, suggesting that autophagy, also, can inhibit other inflammatory pathways.

In this study we also observed elevated levels of sEng secreted by the trophoblast when autophagy was inhibited with bafilomycin, again similarly to aPL. This suggests that the absence of the autophagy process can cause an imbalance in angiogenic factor production and this may be how aPL mediates this effect. However, the sEng response to aPL was inhibited when autophagy was maintained. From the literature, Saito & Nakashima (2013) found that sEng inhibited the number of autophagosomes in extravillous trophoblast cells and reduced LC3B-II expression. Thus, they demonstrated that sEng reduced extravillous trophoblast autophagy response and our results shown that the absence of autophagy enhances sEng production.

In conclusion these results showed that aPL impair trophoblast autophagy which is turn allows inflammasome activation and subsequent IL-1β production, as well as TLR4-mediated IL-8 secretion to occur. In addition, a later induction or recovery of trophoblast autophagy may be triggered by the inflammasome, however, further studies are needed to confirm this.

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Conclusões

5. Conclusões

I- Placentas de gestantes portadoras de pré-eclâmpsia apresentaram ativação do inflamassoma NLRP3 e caspase 1 com aumento de expressão de IL-1β, TNF-α e HMGB1. Esses resultados sugerem que este complexo inflamatório pode estar envolvido na resposta inflamatória exacerbada observada em placenta de gestantes pré-eclâmpticas.

II- Placentas de gestantes com pré-eclâmpsia apresentaram diminuição na resposta autofágica, caracterizada pela menor expressão de LC3B-II e beclin-1 e aumento de expressão do mTOR, sugerindo que esta diminuição pode estar envolvida na patogênese da pré-eclâmpsia.

III- O anticorpo anti-fosfolípide é capaz de reduzir a resposta autofágica em células de trofoblasto extraviloso, que por sua vez permite a ativação do inflamassoma, com secreção de IL-1β e de IL-8 mediada pelo TLR4. Além disso, sugere-se que ocorra um reestabelecimento da autofagia desencadeada pela ativação do inflamassoma.

O presente estudo evidendiou a participação de autofagia e inflamassoma nas patologias da gestação, mostrando que ocorre uma resposta inflamatória exacerbada tanto na pré-eclâmpsia quanto em células trofoblástias de primeiro trimestre induzidas por anticorpo anti-fosfolípide. Além disso, mostrou que o processo autofágico encontra-se deprimido em placenta de gestante com pré-eclâmpsia e em células de trofoblasto induzidas por aPL. Estes resultados revelam que mecanismos complexos estão envolvidos em patologias da gestação e que merecem uma investigação mais aprofundada.

Anexos

Assunto: Fwd: Submission JRI_2016_139 received by Journal of Reproductive Immunology
De: Terezinha Peraçoli (peracoli@ibb.unesp.br)
Para: icweel@yahoo.com.br;
Data: Segunda-feira, 18 de Julho de 2016 11:12

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Title: Inflammasome activation in placentas from pregnant women with preeclampsia
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FACULDADE DE MEDICINA DE BOTUCATU -UNESP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ENVOLVIMENTO DE INFLAMASSOMO E AUTOFAGIA NA FISIOPATOLOGIA DA PRÉ-ECLÂMPSIA

Subprojeto 1: Avaliação de inflamassomo NLRP3 e autofagia em placentas de gestantes portadoras de pré-eclâmpsia

Subprojeto 2: Envolvimento do inflamassomo na imunidade inata e adaptativa em gestantes portadoras de pré-eclâmpsia

Subprojeto 3:Análise do inflamassomo NLRP3 induzido por ácido úrico em monócitos de gestantes portadoras de pré-eclâmpsia

Pesquisador: Maria Terezinha Serrão Peraçoli

Área Temática:

Versão: 2

CAAE: 17748313.2.0000.5411

Instituição Proponente: Departamento de Microbiologia e Imunologia

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

DADOS DO PARECER

Número do Parecer: 349.847

Data da Relatoria: 05/08/2013

Apresentação do Projeto:

Projeto envolvendo 3 subprojetos que são complementares (1 mestrado e 2 doutorad) ser conduzido pela pesquisadora e colaboradores (Ingrid Cristina Weel, José Carlos Peraçolli, Mariana Letícia Matias, Mariana Romão e Vera Therezinha Borges).

Objetivo da Pesquisa:

Subprojeto 1: a)Avaliar a presença de inflamassomo NLRP3 pela detecção de caspase-1, citocinas produzidas (IL-1ß e IL-18) e expressão de HMGB1 e TNF-a em placenta de gestantes normotensas e portadoras de PE; b)Avaliar a ocorrência de autofagia através da análise da expressão das proteínas LC3, Beclin-1 e mTOR em tecido placentário de gestantes normotensas e portadoras de PE.

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Subprojeto 2: a) A concentração das DAMPs (Hsp70, HMGB1, Hialuronan e Ácido Úrico) no plasma; b)O estado de ativação, endógena e induzida pelas DAMPs (Hsp70, hialuronan e urato monossódico), em monócitos de gestantes com PE pela identificação da presença de inflamassomo NLRP3 e sua associação com a produção de IL-1b, TNF-a e IL-18 nessas células; c)O envolvimento das subpopulações de células T (Th1, Th2, Treg e Th17) na fisiopatologia da PE, analisando o perfil de citocinas e fatores de transcrição produzidos por essas subpopulações celulares; d) Correlacionar a ativação do inflamassomo com o perfil das subpopulações de células T.
Subprojeto 3: a)Avaliar o estado de ativação, endógena e induzida por urato monossódico, de monócitos em gestantes portadoras de PE pela presença de inflamassomo NLRP1/NLRP3 e sua associação com a produção de IL-1b, TNF-a e IL- 18 por essas células; b)Correlacionar a concentração plasmática de ácido úrico com a ativação do inflamassomo e produção de citocinas inflamatórias por monócitos de gestantes com PE.

Avaliação dos Riscos e Benefícios:

Riscos:

Subprojeto 1: A coleta da placenta não oferece riscos à gestante, a não ser os riscos relativos ao parto e que não estão relacionados com a pesquisa. Subprojeto 2 e 3: No momento da coleta de sangue pode haver dor da picada de agulha e, raramente, formação de um pequeno hematoma no local.

Benefícios:

Os resultados contribuirão para melhor conhecimento dos mecanismos imunes envolvido na fisiopatologia da PE e poderão, no futuro estabelecer estratégias diferentes de prevenção e tratamento dessa patologia obstétrica.

Comentários e Considerações sobre a Pesquisa:

Trata-se de estudo unicentrico, envolvendo 3 subprojetos (1 Mestrado e 2 Doutorado).

Suprojeto 1: avaliação da placenta de 20 gestantes com PE e 20 normotensas.

Subprojeto2:40 gestantes (20 com PE e 20 normotensas), pareadas por idade gestacional e 20 mulheres saudáveis não grávidas.

Subprojeto 3: 46 gestantes (23 normotensas e 23 PE), pareadas por idade gestacional e 23 mulheres saudáveis não grávidas.

Critério de Inclusão:

Subprojeto 1: Gestantes: Ter gestação única, IG entre 28 e 40 semanas com pré-natal e parto no Serviço de Obstetrícia do HC FMB UNESP.

Subprojeto 2 e 3: Gestantes: Ter gestação única, IG entre 28 e 40 semanas com pré-natal e parto no Serviço de Obstetrícia do HC FMB UNESP.
Mulheres normais, não-grávidas: ter a mesma faixa etária das gestantes e serem doadoras voluntárias do Banco de sangue do Hemocentro da FMB-UNESP, sem diagnóstico de qualquer patologia obstétrica ou clínica.
Critério de Exclusão:
Subprojeto 1, 2 e 3: Apresentar qualquer intercorrência obstétrica ou clínica, com exceção de PE tais como: gestação gemelar, préeclâmpsia anterior, hipertensão crônica, diabetes, doenças renais, má-formação fetal, doenças infecciosas, soropositividade para HIV e uso de
drogas e álcool ou não ter a gestação resolvida na Maternidade do HC FMB-UNESP.
Metodologia de análise dos dados adequada (vide projeto).
Número total de sujeitos: 69 sendo 23 mulheres não grávidas (coleta de sangue); 23 com PE (coleta de sangue e/ou placenta); 23 gestantes normotensas (sangue e/ou placenta).
Orçamento: 366.633.85 (FAPESP).
TCLE: elaborado para as gestantes com e sem pré- eclâmpsia e para as doadoras de sangue do hemocentro, em forma de convite, com explicação sobre a doença e sobre o objetivo da pesquisa.

Considerações sobre os Termos de apresentação obrigatória:

O projeto apresenta os seguintes termos:
- Projetos originais
- TCLE apenas para as gestantes com e sem PE e para as doadoras de sangue do hemocenrtro (TCLE)
- Autorização do superintendente do HC
- Autorização do responsável pelo hemocentro
- Folha de rosto (insituição proponente IB)
- Termo de compromisso de cumprimento da resolução 196/96
- Declaração de relatório final e cronograma de execução
- Declaração de autorização do Chefe de depto da Micro/Imuno
- Declaração de autorização do Chefe de depto da GO

Recomendações:

nenhuma

Conclusões ou Pendências e Lista de Inadequações:

Os autores revisaram o número de sujeitos do estudo (23 não grávidas, 23 grávidas com pre-

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eclâmpsia e 23 gravidas sem pre-eclâmpsia); redigiram um novo TCLE para gravidas com e sem a doença de forma mais explicativa e acrescentaram um novo TCLE para as doadoras de sangue do hemocentro. Também obtiveram a autorização do responsável pelo hemocentro.
Sendo assim, sugiro aprovação do projeto, sem necessidade de envio a CONEP.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Projeto de pesquisa APROVADO em reunião do CEP de 05/08/2013, sem necessidade de envio à CONEP.

BOTUCATU, 05 de Agosto de 2013

Assinador por:
Trajano Sardenberg
(Coordenador)

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unesp

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Botucatu

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE INGRID CRISTINA WEEL, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA GERAL E APLICADA, DO INSTITUTO DE BIOCIÊNCIAS.

Aos 01 dias do mês de setembro do ano de 2016, às 14:00 horas, no(a) Anfiteatro do IBTEC, reuniu-se a Comissão Examinadora da Defesa Pública, composta pelos seguintes membros: Profa. Dra. MARIA TEREZINHA SERRÃO PERAÇOLI - Orientador(a) do(a) Departamento de Microbiologia e Imunologia / Instituto de Biociências de Botucatu - UNESP, Prof. Dr. FRANCISCO LÁZARO PEREIRA DE SOUSA do(a) Departamento de Tocoginecologia / Centro Universitário Lusíada, Profa. Dra. ALESSANDRA PONTILLO do(a) Departamento de Imunologia / Instituto de Ciências Biomédicas - USP, Prof. Dr. ROBERTO ANTONIO DE ARAUJO COSTA do(a) Departamento de Ginecologia e Obstetrícia / Faculdade de Medicina de Botucatu - UNESP, Prof. Dr. DANIELA CARVALHO DOS SANTOS do(a) Departamento de Morfologia / Instituto de Biociências de Botucatu - UNESP, sob a presidência do primeiro, a fim de proceder a arguição pública da TESE DE DOUTORADO de INGRID CRISTINA WEEL, intitulada **Avaliação do inflamassoma NLRP3 e autofagia em placentas de gestantes portadoras de pré-eclâmpsia** . Após a exposição, a discente foi arguida oralmente pelos membros da Comissão Examinadora, tendo recebido o conceito final: APROVADA . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.

Profa. Dra. MARIA TEREZINHA SERRÃO PERAÇOLI

Prof. Dr. FRANCISCO LÁZARO PEREIRA DE SOUSA

Profa. Dra. ALESSANDRA PONTILLO

Prof. Dr. ROBERTO ANTONIO DE ARAUJO COSTA

Prof. Dr. DANIELA CARVALHO DOS SANTOS