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

Giovana Fernanda Cosi Bento

Infecção bacteriana das membranas corioamnióticas *in vitro*: interação microbiana, resposta inflamatória/oxidativa e senescência celular.

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Doutora em Ciências – Área Patologia.

Orientadora: Profa. Dra. Márcia Guimarães da Silva
Coorientadora: Profa. Dra. Jossimara Polettini

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Orientador: Márcia Guimarães da Silva
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UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Botucatu

FMB

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE GIOVANA FERNANDA COSI BENTO, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA, DA FACULDADE DE MEDICINA - CÂMPUS DE BOTUCATU.

Aos 12 dias do mês de abril do ano de 2024, às 14:00 horas, no(a) Anfiteatro Kunie Iabuki Rabello Coelho - Depto. de Patologia - FM/Botucatu - Unesp, realizou-se a defesa de TESE DE DOUTORADO de GIOVANA FERNANDA COSI BENTO, intitulada **Infecção bacteriana das membranas corioamnióticas in vitro: interação microbiana, resposta inflamatória/oxidativa e senescência celular.**. A Comissão Examinadora foi constituida pelos seguintes membros: Profa. Dra. MARCIA GUIMARÃES DA SILVA (Orientador(a) - Participação Presencial) do(a) Depto. de Patologia / FM/Botucatu - Unesp, Prof. Dr. RODOLFO DE CARVALHO PACAGNELLA (Participação Virtual) do(a) Depto. de Tocoginecologia / FCM/Campinas - Unicamp, Profa. Dra. GISLANE LELIS VILELA DE OLIVEIRA (Participação Presencial) do(a) Depto. de Ciências Químicas e Biológicas / IB/Botucatu - Unesp. Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: Aprovada. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. MARCIA GUIMARÃES DA SILVA

Título da Tese: Infecção bacteriana das membranas corioamnióticas in vitro: interação microbiana, resposta inflamatória/oxidativa e senescência celular.

Impacto científico esperado: Validação de um novo modelo de estudo que mimetiza o microambiente uterino *in vivo*, o *feto-maternal interface organ-on-chip* para estudos de infecção bacteriana ascendente do trato genital inferior feminino, permitindo a análise da resposta inflamatória/oxidativa, de vias de sinalização celular e senescência.

Impacto social: Atualmente, o parto pré-termo é considerado um problema de saúde pública mundial. Os resultados e achados desses estudos permitem que estratégias na clínica obstétrica associadas ao parto pré-termo sejam atualizadas, considerando que a infecção bacteriana ascendente está associada à aproximadamente 40% dos partos pré-termo. A contribuição dessa tese se insere nos Objetivos de Desenvolvimento Sustentável (ODS) da ONU na qualidade "Saúde e Bem-estar".

Impacto econômico: A redução da prevalência de parto pré-termo reduz os custos no sistema de saúde envolvidos nas morbidades maternas e neonatais resultantes desse desfecho gestacional adverso.

Dedicatória

Dedico esse trabalho aos meus pais, Claudia e Enzo, que são meu porto seguro, minha inspiração e exemplo.

E aos meus avós, Neusa, Altino (*in memorian)*, Maria (*in memorian)* e Elzo (*in memorian)* que sempre me incentivaram, comemoraram minhas vitórias e se orgulharam da minha caminhada.

“A beleza do caminho está na caminhada.”
(Pe. Lucas Emanuel Almeida)

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A **Deus** e à **Nossa Senhora**,
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Sumário

Resumo

INTRODUÇÃO: A ascensão bacteriana do trato genital inferior feminino para a cavidade amniótica está associada a cerca de 40% do total de partos pré-termo (PPT) espontâneos. O gênero *Ureaplasma* spp. é um dos mais frequentemente isolados do líquido amniótico, seguido de outras espécies como *Streptococcus aureus*, *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius* que também estão associadas a esse desfecho gestacional adverso. A resposta inflamatória tem participação fundamental no desencadeamento do parto pré-termo, aliado ao estresse oxidativo e a senescência e, juntos, esses processos corroboram para a deflagração do parto pré-termo espontâneo. O desenvolvimento de novos modelos *in vitro* apropriados que mimetizam a propagação da inecção *in vivo* é essencial para compreensão da patogênese dos desfechos gestacionais, com ênfase no entendimento de diferentes interações bacterianas. O objetivo desse estudo foi avaliar a regulação da resposta inflamatória e oxidativa em modelos *in vitro* de células que compõe as membranas corioamnióticas com estimulação bacteriana associada ao parto pré-termo, e correlacionar com a senescência e sinalização celular.

METODOLOGIA: Células deciduais, coriônicas trofoblásticas, amnióticas mesenquimais e amnióticas epiteliais imortalizadas foram cultivadas em modelo *in vitro* 2D e modelo de *organ-on-chip*. O *feto-maternal interface organ-on-chip* (FMi-OOC) é um dispositivo microfisiológico que mimetiza a interface materno-fetal composto por 4 câmaras interconectadas por microcanais. A propagação ascendente de *U. parvum* foi confirmada para validação do modelo (Bento et al., 2023). Em seguida, determinamos o impacto da infecção na citotoxicidade, sinalização celular, transição celular e inflamação. O estresse oxidativo foi avaliado em modelo de cultura 2D de células amnióticas mesenquimais e epiteliais com mimetização de infecção polibacteriana.

RESULTADOS: No modelo FMI-OOC, *U. parvum* atravessou o córion e atingiu o epitélio do âmnio após 72 horas, mas não induziu a ativação de quinases de sinalização celular (p38MAPK e JNK) ou a transição celular (epitelial-mesenquimal), independentemente da presença de células imunes. A resposta inflamatória causada por *U. parvum*, determinada pelos níveis de Interleucina (IL)-6, IL-8, 1L-10, TNFα e GM-CSF, foi limitada à interface coriodecidual e não promoveu inflamação na camada amniótica. No modelo de cultura 2D, a peroxidação lipídica e proteica causada pela infecção polibacteriana por espécies anaeróbias facultativas e estritas se limitou às células amnióticas epiteliais após 24 horas, enquanto que a infecção reduziu significativamente

os níveis da capacidade antioxidante total somente nas células amnióticas mesenquimais. **CONCLUSÕES**: O modelo FMI-OCC foi validado para infecções ascendentes com células bacterianas vivas. Os nossos dados corroboram a sugestão que *U. parvum* é pouco imunogênico e não produz alterações inflamatórias maciças na interface feto-materna. Especulamos que a presença de *U. parvum* pode ainda comprometer a interface feto-materna, tornando-a suscetível a outras infecções patogênicas. Além disso, a infecção polibacteriana da camada amniótica está associada ao estresse oxidativo por meio da peroxidação lipídica e proteica, e da redução da capacidade antioxidante.

Palavras-chaves: parto pré-termo; infecção ascendente; inflamação; estresse oxidativo; senescência; *organ-on-chip*.

Abstract

INTRODUCTION: Ascending bacterial infection from the female lower genital tract to the amniotic cavity is associated with 40% of all spontaneous preterm births (PTB). The genus *Ureaplasma* spp. is one of the most frequently isolated from amniotic fluid, followed by other species such as *Streptococcus aureus, Fusobacterium nucleatum, Peptostreptococcus anaerobius* which are also associated with this adverse gestational outcome. The inflammatory response plays a crucial role in triggering preterm birth, along with oxidative stress and senescence, and together these processes corroborate to the onset of spontaneous preterm birth. The development of new *in vitro* models that mimic the spread of infection *in vivo* is essential for understanding the pathogenesis of gestational outcomes, with an emphasis on understanding different bacterial interactions. The aim of this study was to evaluate the regulation of the inflammatory and oxidative response in *in vitro* models of cells which mimetize the fetal membranes stimulated with bacterial species associated with preterm birth, and to correlate this with senescence and cell signaling.

METHODOLOGY: Immortalized decidual, chorionic trophoblastic, amnion mesenchymal, and amnion epithelial cells were cultured in 2D *in vitro* model and *organ-on-chip* model. The feto-maternal interface organ-on-chip (FMi-OOC) is a microphysiological device that mimics the maternal-fetal interface composed of 4 chambers interconnected by microchannels. The ascending propagation of *U. parvum* was confirmed to validate the model (Bento et al., 2023). Next, we determined the impact of infection on cytotoxicity, cell signaling, cell transition, and inflammation. Oxidative stress was assessed in a 2D culture model of amniotic mesenchymal and epithelial cells mimicking polybacterial infection.

RESULTS: In the FMI-OOC model, *U. parvum* transversed the chorion and reached the amnion epithelial cells after 72 hours, but did not induce the activation of cell signaling kinases (p38MAPK and JNK) or cell transition (epithelial-mesenchymal), regardless of the presence of immune cells. The inflammatory response caused by *U. parvum*, determined by the levels of Interleukin (IL)-6, IL-8, 1L-10, TNFα and GM-CSF, was limited to the choriodecidual interface and did not promote inflammation in the amniotic layer. In the 2D culture model, lipid and protein peroxidation caused by polybacterial infection by strict and facultative anaerobic species was limited to epithelial amniotic cells after 24 hours, while infection significantly reduced levels of total antioxidant capacity only in mesenchymal amniotic cells.

CONCLUSIONS: The FMI-OCC model was validated for ascending infections with live bacterial cells. Our data corroborate the suggestion that *U. parvum* is poorly immunogenic and does not produce massive inflammatory changes at the feto-maternal interface. We speculate that the presence of *U. parvum* may compromise the feto-maternal interface, making it susceptible to other pathogenic infections. In addition, polybacterial infection of the amniotic layer is associated with oxidative stress through lipid and protein peroxidation and reduced antioxidant capacity.

Keywords: preterm birth; ascending infection; inflammation; oxidative stress; senescence; *organ-on-chip*.

Capítulo I

Revisão de Literatura

1. A resposta imunológica na gestação

Os mecanismos imunológicos envolvidos na gestação contribuem para o estabelecimento de uma interface materno-fetal que participa do desfecho gestacional favorável ou adverso (1-3). Para que uma gestação alcance o termo, são necessários mecanismos imunológicos específicos em cada trimestre, com presença fundamental de células imunes para a facilitação e proteção da gestação (4). Um movimento orquestrado entre a invasão das células trofoblásticas e imunológicas é essencial para que a gestação tanto se desenvolva quanto se mantenha (5). Nesse sentido, a interface materno-fetal, que desempenha funções importantes ao longo de todo o desenvolvimento gestacional, é constituída por diferentes células imunes, como as células T, células T reguladoras, além de macrófagos, células dendríticas, células *natural killers (*NK), entre outras (6). A gravidez e o crescimento fetal representam um desafio não trivial para o sistema imune materno. Desde os estágios iniciais da implantação e formação da placenta até a evolução do parto vaginal bem-sucedido, os mediadores imunes maternos devem se apresentar finamente modulados para se evitar desfechos gestacionais adversos e até mesmo a perda gestacional. Em resumo, a forma como muitos mediadores imunes se expressa no cenário da gestação terá impacto na saúde materna e fetal. As células T reguladoras (Treg - $CD4^{+}CD25^{+}Foxp3^{+}$) desempenham um papel importante desde o momento da implantação até a manutenção da gestação (7). Esse tipo celular tem participação fundamental na tolerância imunogênica e também na homeostase dos tecidos (8,9). Além disso, a população de células Treg efetoras da decídua está aumentada no 3º trimestre de

gestação em comparação com o 1º trimestre (8).

De forma geral, o primeiro trimestre da gestação é caracterizado predominantemente pelo perfil Th (T*helper)1* de expressão de citocinas, para estimular reparos do evento da implantação. Vários genes da resposta imune foram considerados como reguladores do período de implantação, incluindo o fator declínio de aceleração (DAF - *decay accelerating factor*), indoleamina 2,3 dioxigenase (IDO), Interleucina (IL-)15, receptor antagonista de IL-15, fator regulador de interferon-1 (IRF-1), linfotoxina, transcriptase 2 associado a NK (NKAT2) e NKG5. Esses genes estão envolvidos em um conjunto importante de funções, incluindo a promoção da proliferação das células NK uterinas, quimiotaxia, inibição da atividade citolítica das células NK, inibição do crescimento celular (células T e patógenos) e inibição da via clássica do sistema complemento (7). Nesse momento da gestação, é importante que o mecanismo de tolerância materno-fetal aconteça de forma adequada. No primeiro trimestre de gestação, 70% a 80% dos linfócitos presentes na mucosa uterina são linfócitos granulares grandes, que representam menos de 1% da população dos linfócitos do sangue periférico. Embora esses linfócitos sejam fenotipicamente e morfologicamente classificados como células NK, são menos citotóxicos que as células NK do sangue periférico (8). As células NK uterinas constituem 70% dos linfócitos da decídua humana, em contraste com as células NK do sangue periférico, que são menos de 15% dos linfócitos circulantes (9). Além disso, esses linfócitos uterinos proliferam, ativam-se na mucosa e são fonte de citocinas como o fator inibidor de leucemia (*leukemia inhibitory factor* - LIF) e fator estimulador

de colônia de macrófagos (*macrophage-colony stimulating factor* - M-CSF), fator de necrose tumoral (TNF), interferon-g (INF-g) e IL-10, as quais têm importante papel na implantação e desenvolvimento da placenta (7).

Já no segundo trimestre da gestação espera-se o perfil Th2, que tem como característica a expressão de citocinas, como IL-4, IL-5, IL-10 e IL-13, para facilitar a manutenção da gestação após o período de implantação. Esse perfil também está associado à produção de anticorpos pelas células B (10). A regulação e diferenciação das células Th2 acontece através das células dendríticas (CDs). Estudos mostram que as CDs desempenham papel importante nesse contexto, visto que a expressão de moléculas coestimuladoras OX40L e o ligante Notch (*Notch ligand Jagged 1)* promovem o *priming* de células Th2 (11). A expressão dos marcadores CD200 e B7-H1 em CDs atinge seu pico no segundo trimestre de gestação, desempenhando papel importante de imunorregulação nesse período (12). É no segundo trimestre de gestação que os neutrófilos estão em maior proporção quando comparado aos outros trimestres e ao período pós-parto. Já as células T $CD4^+$ e as células T $CD8^+$ têm a menor proporção nesse trimestre gestacional (13). Genes codificadores de proteínas têm expressão associada ao segundo trimestre de gestação. A família dos carreadores de solutos orgânicos (*solute carrier Family* – SLC), composta por diferentes tipos de transportadores e presente na membrana celular, tem sua expressão aumentada associada ao segundo trimestre de gestação. Além disso, genes como *CD55*, associado ao sistema complemento, *IFNGR1*, associado à via do interferon, *NLRP6*, associado à ativação do inflamassoma, também tiveram associação com esse trimestre (13).

Juntos, esses achados corroboram com a função de manutenção da gestação marcado pelo perfil Th2 de expressão de citocinas.

Finalmente o terceiro trimestre volta a ter o perfil Th1 para contribuir na deflagração do trabalho de parto, definido na literatura como uma síndrome inflamatória (14). A quantidade de neutrófilos nesse período se assemelha aos níveis do primeiro trimestre de gestação (13). Já os genes associados a esse período têm principalmente a função de resposta às infecções bacterianas e participação no processo de resposta imune efetora. Além disso, genes que codificam proteínas ribossômicas, genes do sistema complemento *C1QA/B/C* e gene *CD24*, expresso em granulócitos maduros e que modulam os sinais de crescimento e diferenciação dessas células, também estão associados ao último trimestre de gestação(13). Nesse contexto, os danos ao DNA de leucócitos são maiores no último trimestre de gestação, reforçando a associação da síndrome inflamatória, juntamente com o estresse oxidativo para o desfecho gestacional (15). A análise de *clusters*/família de miRNA de gestações no terceiro trimestre mostra que *clusters* associados a resposta imune inata e adaptativa, diferenciação celular e supressão de tumores estiveram *upregulated* quando comparado ao primeiro trimestre gestacional (16). O evento do terceiro trimestre considerado como uma síndrome inflamatória é reafirmado pela diminuição dos níveis de IL-10 que pode levar à ativação de células miometriais, culminando no início da contratilidade uterina (17).

2. Parto pré-termo

Quando um ou mais dos inúmeros processos que fazem parte da manutenção da gestação envolvendo diferentes sistemas (endócrino, imunológico e nervoso) é afetado, a consequência pode ser o parto pré-termo , definido por aquele que acontece antes das 37 semanas completas (259 dias) de gestação (18). O parto pré-termo é mundialmente considerado a principalmente causa de morbidade e mortalidade neonatais (19). No contexto mundial, 15 milhões de bebês nascem prematuros todo ano, correspondendo entre 5-18% do total de gestações, a depender a localidade (19, 20). Em 2014, 10,6% do total de gestações no mundo foram pré-termo, variando regionalmente com 13,4% na África e 8,7% na Europa (21). Já na América Latina, a prevalência foi de 9,8% do total de gestações. No Brasil, a taxa de partos pré-termo foi de 11,2%, correspondendo a 2,3% da proporção mundial de casos (21). Esses dados corroboram com a preocupação existente hoje na clínica obstétrica para que as taxas desse desfecho gestacional adverso diminuam, já que ao longo dos anos as taxas não têm sofrido alterações, podendo até aumentar a incidência em algumas regiões (19). Para que isso aconteça, é necessário estudar com precisão e meticulosidade a etiologia e os mecanismos envolvidos nesse cenário. Os partos pré-termo podem ser espontâneos ou por indicação médica devido às condições maternas ou fetais, sendo que os espontâneos contabilizam 75% do total de partos pré-termo, e incluem a rotura prematura de membranas pré-termo (RPM-PT) (22, 23).

É sabido que a etiologia do parto pré-termo é multifatorial, e alguns dos fatores sabidamente associados são o histórico anterior de parto pré-termo, fatores genéticos, condições maternas como diabetes, hipertensão, asma; hábitos

comportamentais como o uso de álcool, drogas e tabagismo; obesidade e desnutrição; etnia, e outro fatores como mostrado na Tabela 1(24-26) (Tabela 1). Somado à esses fatores, destaca-se a infecção bacteriana ascendente do trato genital inferior para a cavidade amniótica como responsável por 40% do total de casos de parto pré-termo (27).

Tabela 1. Fatores de risco associados ao Parto Pré-termo.

ETIOLOGIA PARTO PRÉ-TERMO	
FATORES DEMOGRÁFICOS E HÁBITOS	**CONDIÇÕES MATERNAS**
Idade materna [24 25 26]	Hipertensão [24 26]
Etnia [24 25 26 27]	Diabetes [24 26]
Nível de escolaridade [24 25]	Asma [24 26]
Tabagismo [24 25 26]	Infecção intra-amniótica [24 25 26]
Uso de álcool e drogas [24 26]	Histórico de parto pré-termo [24 25 27]
Exposição à poluentes [24 26]	Infecção do trato urinário [24 25 26 27]
Saúde mental [24 25 27]	Genética [24 26 27]
Status socioeconômico [24 25 26]	Infecções sexualmente transmissíveis [24 25 27]
	Índice de massa corporal (IMC) [25 26]
	Disbiose vaginal [24 25]
	Histórico de aborto [24 25]

Ref: (24) Menon et al., 2008; (25) Cobo et al., 2020; (26) Mead et al., 2023; (27) Goldenberg et al., 2008.

O modelo de infecção ascendente do trato genital inferior para a cavidade amniótica mais aceito atualmente é o descrito por Kim et al. (2010), que consiste na ascensão bacteriana advinda da microbiota vaginal, instalação em uma região focal cervical, seguido pela proliferação no líquido amniótico para subsequente infecção amniótica (Figura 1). Sendo o parto considerado um processo

inflamatório, sabe-se que estímulos patogênicos podem estimular a resposta imune decidual de forma não fisiológica e não modulada, com recrutamento e ativação leucocitárias local, resultando em resposta inflamatória nas membranas corioamnióticas, denominada de corioamnionite histológica (28, 29).

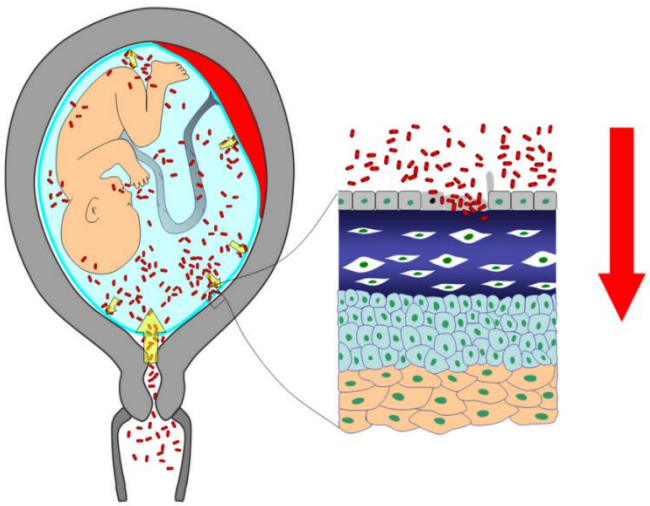

Figura 1. Modelo de infecção bacteriana ascendente do trato genital inferior para a cavidade amniótica (Kim et al. (2010).

3. Estrutura morfofuncional das membranas fetais

As membranas fetais, ou membranas corioamnióticas, são barreiras física e imunológica que envolvem o feto na cavidade amniótica (30). As membranas são constituídas de diferentes camadas celulares e por componentes da matriz extracelular (MEC), e também desempenham as funções de manter as condições ideais do líquido amniótico e de ser barreira imunológica, sendo essa última função confirmada pela presença de receptores *Toll-like* (TLR) no epitélio amniótico (31). Com relação à estrutura histológica das membranas fetais, o cório é a camada mais externa, constituído pelas camadas celular e reticular e pela membrana basal (32). Já a camada mais interna, o âmnio, é constituído por

monocamada de células epiteliais cuboides ou colunares juntamente com tecido rico em colágeno, intercalada por células mesenquimais (33). É o cório que está em contato com a decídua, camada formada a partir do endométrio e que é parte da mucosa uterina.

Nesse sentido, o termo interface materno-fetal compreende as membranas fetais e a decídua, tendo assim a representação dos lados materno e fetal (Figura 2) (34). Cada tipo celular que compõe a interface desempenha funções específicas e características individuais que, em conjunto, reforçam a importância dessa estrutura ao longo da gestação. A interface materno-fetal tem sido muito estudada no contexto dos desfechos gestacionais adversos (35-39). Cada tipo celular que compõe a interface está envolvido na produção de diferentes mediadores, que estão envolvidos em diferentes mecanismos e sinalizações celulares. As células epiteliais, por exemplo, podem expressar antígeno leucocitário humano G (HLA-G), envolvido na apresentação de antígenos e reforçando a função imunomoduladora dessa camada (40). Já as células amnióticas mesenquimais secretam diferentes tipos de colágeno que então farão parte da matriz extracelular, que também é composta por fibronectina e proteoglicanos (41). Além disso, as células coriônicas trofoblásticas são responsáveis por secretar hormônios, como progesterona e gonadotrofina coriônica humana (*human chorionic gonadotropin* - HCG)(42). As células da decídua, considerada o tecido uterino materno, estão intimamente relacionada com a resposta imune devido a presença de células imunes, como macrófagos (43). Além dos fatores de crescimento secretados por esse compartimento, como o fator de crescimento endotelial vascular (*Vascular*

endothelial growth factor -VEGF) e o fator de crescimento placentário (*Placental growth factor* - PLGF), citocinas e quimiocinas também são secretadas por essas

Figura 2. Estrutura histológica das membranas fetais.

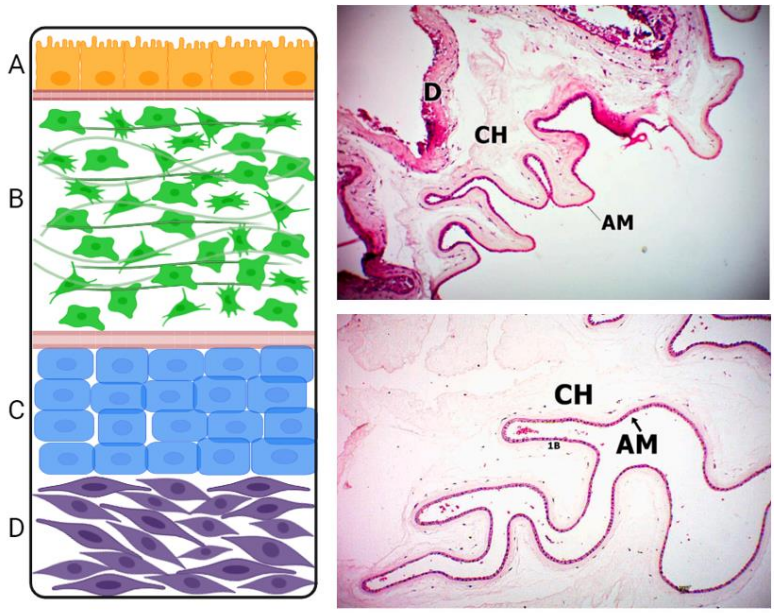

Figura 2. Estrutura histológica das membranas fetais. À esquerda, esquema produzido no software BioRender, apresentando os estratos histológicos que compõem as membranas fetais. A: Células Amnióticas Epiteliais (laranja), B: Células Amnióticas Mesenquimais (verde), C: Células Coriônicas Trofoblásticas (azul), D: Células deciduais (roxo). À direita, cortes histológicos das membranas feitas retirado do estudo de Singoei et al. (2021). AM: Camada Amniótica, CH: Camada Coriônica, D: Camada Decidual.

células. Sendo que esses dois últimos também são secretados pelos outros tipos celulares da interface materno- fetal (44).

4. Infecção ascendente do trato genital inferior feminino para a cavidade amniótica

São diversas as espécies bacterianas que podem ascender o trato genital

inferior feminino e causar infecção intra-amniótica, sendo esses microrganismos comumente identificados na microbiota vaginal, como por exemplo *Gardnerella vaginalis, Streptococcus agalactiae, Bacteroides fragilis, Prevotella bivia, Sneathia sanguinegens*, *Mycoplasma hominis* e *Ureaplasma* spp. (29, 45-48). Na maioria dos casos, a infecção ascendente é polimicrobiana, sendo os micoplasmas genitais, *M. hominis, U. urealyticum* e *U. parvum* as espécies mais comumentes identificadas no líquido amniótico de casos de parto pré-termo (45, 47). Considerando a característica polimicrobiana da infecção ascendente, é fundamental que a complexidade dos aspectos microbiológicos e os mecanismos de patogênese sejam estudados, visto que interações entre espécies podem culminar em desfechos clínicos distintos (49).

Diversos trabalhos na literatura têm associado os micoplasmas genitais com o parto pré-termo e morbidades neonatais, tanto na infecção do líquido amniótico (45, 50), como também na colonização vaginal (51, 52) e cervical (53). Porém, em revisão sistemática publicada pelo nosso grupo de pesquisa foi observado que apesar da associação da presença de *Ureaplasma* spp. com trabalho de parto pré-termo e com o parto pré-termo, não foram observadas associações desse gênero com marcadores inflamatórios, considerados a chave principal para o desencadeamento do trabalho de parto. Existe atualmente uma heterogeneidade na literatura com relação ao momento do diagnóstico, tipo de amostra usada para detecção e método de diagnóstico. Além disso, carga bacteriana e localização da infecção também são fatores que corroboram para que o papel dos micoplasmas genitais, no contexto do parto pré-termo, ainda seja inconclusivo (54).

A ambiguidade na associação de *Ureaplasma* spp. com resultados adversos na gestação foi observada não só nesse estudo, como também em outra revisão sistemática assinada por Jonduo et al. (55), que descreveram a falta de controle de fatores confundidores na maioria dos estudos incluídos na metanálise. Além disso, a maioria dos estudos incluídos apresentou análises univariadas dos dados e heterogeneidades similares às encontradas na revisão de Noda-Nicolau et al. (54). Outro estudo mostrou que amostras de líquido amniótico de pacientes que tiveram o desfecho de parto pré-termo foram negativas para detecção de *Ureaplasma* spp. pela técnica de PCR, porém associadas com outros fatores de risco, como hipertensão materna, baixo peso ao nascer do recém-nascido (56). Em suma, é fundamental que estudos sejam delineados para diminuir o *gap* existente no conhecimento do potencial dos micoplasmas genitais no contexto dos desfechos gestacionais adversos.

É importante também ressaltar o impacto causado pela infecção de outras espécies encontradas na microbiota vaginal no contexto do parto pré-termo. Nesse sentido, a infecção por *S. agalactiae* foi associada significativamente com o parto pré-termo (57-59) e com outros desfechos adversos, como a sepse neonatal precoce (59, 60) enquanto que a espécie *Staphylococcus aureus* foi associada à corioamnionite histológica e ao parto pré-termo (61, 62). *Fusobacterium nucleatum,* considerado um patógeno oral, foi identificado em amostras de casos de parto pré-termo (63), e a translocação da cavidade oral para o microambiente uterino já foi documentada (64). Essa espécie também já foi associada a casos de sepse neonatal (65) e ao óbito fetal (66). O gênero *Bacteroides* spp., sabidamente

associado a vaginose bacteriana, também já foi associado ao trabalho de parto pré-termo (45, 67, 68). Por fim, outra espécie identificada em amostras de líquido amniótico de casos de parto pré-termo é *Peptostreptococcus anaerobius* (45, 68).

Nesse sentido, apesar da infecção bacteriana ascendente ser a principal causa do parto pré-termo, as diferentes interações bacterianas e espécies envolvidas nesse processo culminam na complexidade tanto dos mecanismos patogênicos quanto nas interações com o hospedeiro. Ainda existem questões a serem elucidadas sobre o tema e por isso se fazem necessários modelos de estudos robustos para tanto.

5. Mecanismos envolvidos na infecção ascendente e o parto pré-termo

5.1. Inflamação

A deflagração do trabalho de parto pré-termo e a infecção bacteriana ascendente envolvem diferentes mecanismos e processos. A invasão microbiana da cavidade amniótica gera resposta inflamatória local que é caracterizada primeiramente pela presença de infiltrado leucocitário, tanto da resposta imune inata, quanto da adaptativa (69-72). Ainda nesse sentido, existe um aumento dos níveis de citocinas, quimiocinas e prostaglandinas (73-75). São os neutrófilos e macrófagos/monócitos os principais componentes do sistema imune envolvidos nos casos de infecção intra-amniótica, sendo esses últimos responsáveis pela produção e liberação de citocinas pró-inflamatórias, como IL-1α e β, IL-6, IL-8 e TNF-α (69, 70, 76, 77), entre outras. Entre a longa lista de citocinas, algumas se destacam no cenário do parto pré-termo, como a IL-6 e a IL-22 no contexto da

inflamação intra-amniótica que ocorre após infecção da cavidade (71, 77). Já a *High-Mobility Group Box 1* (HMGB1), proteína que funciona como padrão molecular associado a danos (DAMP) induziu resposta pró-inflamatória nas membranas fetais através do aumento dos níveis de IL-6, TNF-α e vias de sinalização associadas a inflamação fatores de transcrição como oNFκB (78). Não somente as citocinas estão envolvidas na deflagração do parto pré-termo, como também são consideradas por diversos grupos de pesquisa como biomarcadores desse desfecho gestacional adverso (79-81). Entre as diversas citocinas envolvidas nesse contexto, a IL-6 se destaca entre os especialistas da área devido a presença de diferentes receptores que podem se ligar a ela, culminando em perfil pró- ou anti-inflamatório (82). Porém, os resultados encontrados dessas citocinas ainda são controversos. Conde-Agudelo et al. (83) ao analisar 30 biomarcadores, incluindo a IL-6, concluiu que nenhum deles tinha o potencial para ser considerado biomarcador para o parto pré-termo.

Os TLRs, que são fisiologicamente expressos em células endoteliais e imunes da interface materno-fetal, podem ser ativados por DAMPS e padrões moleculares associados a patógenos (PAMPs), e é essa ativação que pode desencadear uma cascata de sinalização intracelular, que culmina na ativação de inflamassoma e subsequente liberação de quimiocinas e citocinas(77). Essa liberação resulta em um novo recrutamento de novas células imunes e ativação de vias de sinalização celular. Todos esses eventos corroboram para que ocorra contratilidade uterina, esvaecimento do colo uterino e rotura das membranas, processos que desencadeiam o trabalho de parto (76, 77, 84).

5.2. Estresse oxidativo e senescência

O estresse oxidativo é definido pelo desequilíbrio do estado redox, ou seja, o desequilíbrio entre o acúmulo de espécies reativas de oxigênio (EROs) e espécies reativas de nitrogênio (ERNs) e a capacidade antioxidante. Esse processo ocorre em células e tecidos, sendo que a presença de EROs faz parte de processos fisiológicos, como por exemplo a sinalização celular (85). Porém, esse processo também pode levar ao encurtamento do telômero por meio da resposta a danos do DNA (86). No contexto da gestação, o estresse oxidativo acontece após 37 semanas de gestação devido ao aumento da atividade metabólica do feto que leva ao envelhecimento da placenta. Já no cenário do trabalho de parto pré-termo, a ocorrência do estresse oxidativo leva ao encurtamento do telômero precoce que culmina no parto pré-termo (87).

Existe uma relação entre o estresse oxidativo e a senescência, definida pela parada do ciclo celular de forma irreversível, que pode ser causada por diferentes mecanismos, sendo o encurtamento de telômeros um deles (88). A senescência precoce da decídua pode levar ao óbito fetal e ao parto pré-termo (87), e assim como o estresse oxidativo, a senescência é um processo fisiológico envolvido no contexto da gestação. Porém, quando ocorre antes do momento ideal, está associada aos desfechos adversos (89). Estudo com cortes histológicos das membranas corioamnióticas mostrou que marcadores de senescência celular, como o p53 e p21 estavam aumentados em casos de parto pré-termo e RPM-PT (90). Além disso, as células senescentes apresentam mudanças em seu fenótipo, e como elas seguem metabolicamente ativas, a secreção de fatores de crescimento e

citocinas continua ocorrendo. Juntos, a secreção desses fatores é denominada de fenótipo secretório associado à senescência, ou SASP (91, 92), com aumento da expressão de IL-1α, IL-1β, IL-6, IL-8 e TNF-α em células senescentes quando comparado com células normais (93, 94).

A proteína-quinase ativada por mitógeno (p38 MAPK) é uma das responsáveis pelo dano oxidativo causado pelo estresse oxidativo (95), sendo também ativada por citocinas pró-inflamatórias, como a IL-1 (96). Estudos demonstraram o papel da p38 MAPK em vários estágios de eventos reprodutivos, porém, os sinais que causam sua indução e ativação, assim como sua expressão ainda não estão completamente elucidados (27). Sabe-se que tanto a p38 MAPK quanto a Jun N-terminal kinase (JNK) estão envolvidas no trabalho de parto a termo e no pré-termo em mecanismos cruciais, como a regulação de genes de citocinas inflamatórias e apoptose (97, 98).

6. Modelos de estudos

6.1. Modelos *in vitro* e animais

Os trabalhos com modelos *in vitro* e modelos animais, como o de roedores por exemplo, são os mais utilizados mundialmente para o estudo dos desfechos gestacionais adversos. Conhecimentos importantes que possibilitaram o avanço no entendimento da fisiopatologia das complicações gestacionais, em especial, sobre a infecção bacteriana ascendente. Modelos *in vitro* podem incluir tanto a cultura de tecidos da interface materno-fetal, quanto a cultura de células que compõem esse microambiente. Estudo utilizando modelo de cultura de tecidos das

membranas fetais mostrou que o contexto polimicrobiano é fundamental para determinar a potência das interações de diferentes espécies na intensidade da resposta inflamatória na interface materno-fetal (38). Também foi possível investigar através de estudos de cultura celular *in vitro* os efeitos do parto pré-termo em doenças pulmonares no feto (99), o potencial terapêutico de moléculas com ação imunomoduladora (100) e o impacto de exossomos na deflagração da resposta inflamatória desse desfecho (101). Em conjunto, existe uma quantidade considerável de estudos na literatura que fazem o uso de modelos experimentais *in vitro*.

Os modelos animais incluem o uso de roedores, primatas, entre outros. Utilizando diferentes modelos e *designs* experimentais de roedores, com o objetivo de avaliar a resposta inflamatória e o desfecho do parto pré-termo, estudos demonstraram associação significativa com a infecção/inflamação intra-amniótica (102-106). O trabalho de parto pré-termo foi associado a níveis de IL-1β e TNF-α (103, 107, 108) em modelos de roedores. Modelos experimentais utilizando primatas também foram criados não somente para estudar o parto pré-termo, como também outros desfechos gestacionais adversos (109-111). Estudo de Kelleher et al. (112) investigou o efeito da infecção intra-amniótica por *U. parvum* na função hemodinâmica e cardíaca, e observou o aumento do comprometimento da artéria pulmonar fetal na presença da infecção.

Porém, apesar desses modelos serem amplamente usados, todos contam com limitações. Os modelos de roedores não mimetizam a estrutura histológica da cavidade humana. Já os modelos de cultura 2D *in vitro* não conseguem mimetizar

interações entre as células, especialmente quando se trabalha com tipos de células diferentes e de interfaces diferentes. Já os modelos de primatas são restritos a instituições de pesquisa específicas, têm um custo elevado para execução, além da aprovação no conselho de ética ser extremamente rigorosa.

6.2. Cultura 3D e *organ-on-chip*

Para superar essas limitações, o desenvolvimento de modelos que mimetizem o ambiente *in utero* são fundamentais para o melhor entendimento dos mecanismos envolvidos na interface materno-fetal que levam a desfechos gestacionais adversos, uma vez que revelam que os tecidos gestacionais, especialmente o epitélio amniótico e as células coriônicas e deciduais, respondem ao estímulo bacteriano com aumento de mediadores da resposta imune inata (113, 114).

Avanços recentes em modelos de cultura 3D e modelos microfisiológicos, como os dispositivos *organ-on-chip* (OOC), ajudam a mimetizar diferentes órgãos intrauterinos, possibilitando o cultivo de diferentes tipos celulares interconectados por microcanais, mimetizando um sistema de órgãos como visto no útero (115, 116). Existem modelos de OOC para diferentes órgãos do corpo humano (117) e na literatura já estão estabelecidos modelos de OOC de pulmão (118, 119), coração (120, 121), rim (122, 123), fígado (124, 125), entre outros.

O *feto-maternal interface organ-on-chip* (FMiOOC) foi desenvolvido para recriar ambos os lados, materno (decídua) e fetal (âmnio e cório) da interface materno-fetal (126). Esse OOC é composto por 4 câmaras, interligadas por 24

microcanais, sendo que cada câmara é composta por um tipo de célula da interface materno fetal (Figura 3). Fazem parte do FMi-OOC as células deciduais,

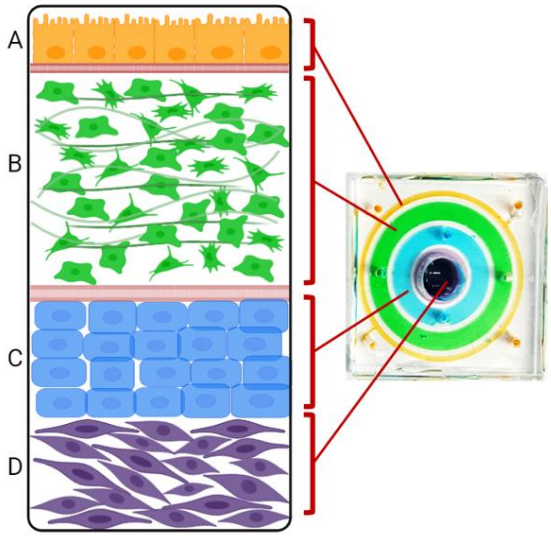

Figura 3. Esquema da estrutura do *feto-maternal interface organ-on-chip* (FMi-OOC). O dispositivo é composto por quatro câmaras circulares conectadas por microcanais. As câmaras correspondem ao seguinte: câmara decidual (roxo- D), câmara de trofoblasto do córion (azul - C), câmara mesenquimal do âmnio (verde - B) e câmara epitelial do âmnio (laranja - A). O desenho esquemático foi criado usando o software BioRender.

coriônicas trofoblásticas, amnióticas mesenquimais e amnióticas epiteliais.

Esse modelo foi usado anteriormente para testar alterações patogênicas causadas por constituintes da parede bacteriana (lipopolissacarídeos [LPS]) (126), produtos químicos ambientais na interface materno-fetal (127) e a farmacocinética de medicamentos (128). Esses dispositivos foram previamente validados por meio de estudos em modelos animais e resultados de estudos anteriores mostram a boa funcionalidade desse OOC, que consegue mimetizar a interface materno-fetal que existe *in vivo*, além de possibilitar o estudo de desfechos gestacionais adversos. Em suma, esse modelo é considerado uma alternativa aos métodos já existentes para estudar a infecção ascendente das

espécies bacterianas do trato genital inferior feminino para a cavidade amniótica.

Diante do exposto, estudos sobre diferentes espécies bacterianas associadas ao parto pré-termo assim como os mecanismos de inflamação e estresse oxidativo envolvidos nesse processo, são fundamentais para que estratégias de prevenção e redução da prevalência desse desfecho gestacional adverso sejam desenvolvidas. Além disso, o desenvolvimento de estudos utilizando modelos que permitam que esse cenário seja mimetizado *in vitro*, possibilita o melhor entendimento dos mecanismos envolvidos. O modelo FMI-OOC possibilita estudos robustos acerca da infecção bacteriana ascendente, assim como a análise da resposta inflamatória e vias de sinalização celular associadas ao parto pré-termo.

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Capítulo II

Capítulo de Livro 1

Capítulo de livro aceito para publicação na revista *Maternal Placental Interface: Methods and Protocols (Springer).*

Title: Feto-maternal interface organ-on-chip: a new technology to study ascending infection.

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Abstract

Modeling human pregnancy is challenging as two subjects, the mother, and fetus must be evaluated in tandem. To understand pregnancy, parturition, and adverse pregnancy outcome, the two feto-maternal interfaces (FMi) that form during gestation (i.e., the placenta and fetal membrane) need to be investigated to understand their biological roles and organ dysfunction can lead to adverse outcomes. Adverse pregnancy outcomes such as preterm rupture of the membranes, spontaneous preterm birth, pre-eclampsia, intra-uterine growth restriction, and gestational diabetes rates are on the rise worldwide, highlighting the need for future studies and a better understanding of molecular and cellular pathways that contribute to disease onset. Current *in vivo* animal models nor *in vitro* cell culture systems can answer these questions as they do not model the function or structure of human FMis. Utilizing microfabrication and soft-lithography techniques, microfluidic organ-on-chip (OOC) devices have been adapted by many fields to model the anatomy and biological function of complex organs and organ systems within small *in vitro* platforms.

These techniques have been adapted to recreate the fetal membrane FMi (FMi-OOC) using immortalized cells and collagen derived from patient samples. The FMi-OOC is a four-cell culture chamber, concentric circle system, that contains both fetal (amniochorion) and maternal (decidua) cellular layers and has been validated to model physiological and pathological states of pregnancy (i.e., ascending infection, systemic oxidative stress, and maternal toxicant exposure). This platform is fully compatible with various analytical methods such as microscopy and biochemical analysis. This protocol will outline the fabrication, cell loading, and utility of this device to model ascending infection-related adverse pregnancy outcomes.

Keywords

Organ-on-chip; feto-maternal interface; ascending infection; cell culture; decidual cell; chorion trophoblast cell; amnion mesenchymal cell; amnion epithelial cell.

1. Introduction

There is a challenge in the obstetrics field in finding the appropriate model to study physiology of pregnancy. To understand the maintenance of pregnancy and mechanisms of parturition both the mother and fetus must be evaluated together. Biological pathways and organ roles of the feto-maternal interface that form during gestation need to be investigated to better understand pregnancy and adverse outcomes. Preterm birth, preterm rupture of the membrane spontaneous, pre-eclampsia, and gestational diabetes are some of the adverse outcomes whose rates are increasing worldwide ***(1-3)***. Models that can improve our understanding of the molecular and cellular mechanisms of pregnancy and parturition, and feto-maternal contributions, are needed to expedite the drug discovery and testing needed to prevent/treat adverse events during pregnancy.

However, current *in vivo* animal models or *in vitro* cell culture studies are not the best model to answer these questions, either because of the anatomy of small animals, or the difficulty to mimic exactly what happens *in vivo*. The biomimetic, microfluid organ-on-chip (OOC) devices, utilizing microfabrication and soft-lithography techniques, recreates *in vivo* organs using *in vitro* cell cultures with different types of cells, mimicking the structure and functions in an organ-level meaning ***(4)***. There are several types of OOC that have been used in the research field: liver-on-a-chip BI ***(5)***, vessel-on-a-chip ***(6)***, kidney-on-a-chip ***(7)***, among others. The use of OOC gives the possibility to study intercellular interactions creating a microenvironment with interconnected channels. There are some models of OOC to study the placenta ***(8-10)***. However, it is essential to have a

model that contains both maternal and fetal sides to model pathological conditions like ascending infection during pregnancy, a major risk associated with preterm birth and preterm premature rupture of the membranes.

The feto-maternal interface organ-on-chip (FMi-OOC) has been developed using cells and collagen derived from patient samples ***(11-14)***. The FMi-OOC is a four-cell culture chamber, concentric circle system, that contains both fetal (amniochorion) and maternal (decidua) cellular layers and has been validated to model physiological and pathological states of pregnancy (i.e., ascending infection, systemic oxidative stress, and maternal toxicant exposure). Each cell chamber is 250μm in height and the width corresponds to the thickness of each layer *in vivo.* The device contains four types of cells: decidual, chorion trophoblast, amnion mesenchymal, and amnion epithelial (Fig. 1), and it is compatible with different analytical methods, including microscopy and protein/biochemical analysis.

To model ascending infection and study the effect of different microorganisms, here we describe how to establish an FMi-OOC device for this purpose. This protocol will outline: the fabrication of the device, preparation for experiments, cell seeding, treatments, and collection of endpoints according to the experiment design.

2. Materials

- Poly(dimethylsiloxane) (PDMS) pre-polymer: Sylgard 184 Silicone, Curing Agent 1:10 Elastomer Base ratio

- 25% Type IV basement membrane collagen Matrigel in Dulbecco's Modified Eagle Medium (DMEM)/F12 serum-free media
- Isopropyl alcohol (IPA)
- Phosphate-buffered saline (PBS)
- Amnion epithelial cells (AEC) media: KSFM supplemented with bovine pituitary extract (30 μg mL), epidermal growth factor (0.1 ng mL), CaCl2 (0.4 mM), and primocin (0.5 mg mL)
- Amnion mesenchymal cells (AMC)/ Decidual cells (DEC) media: DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% amphotericin B
- Chorion trophoblast cells (CTC) media: DMEM/F12 supplemented with 0.20% FBS, 0.1 mM β-mercaptoethanol, 0.5% penicillin/streptomycin, 0.3% bovine serum albumin (BSA), 1× Insulin-Transferrin-Selenium-Ethanolamine (ITS-X), 2 μM CHIR99021, 0.05 μM A83-01, 1.5 μg/mL L-ascorbic acid, 50 ng/mL−1 epithelial growth factor, 0.08 mM Valproic Acid (VPA), and 1× Y27632 (Rock inhibitor)
- Radioimmunoprecipitation assay buffer (RIPA) buffer: RIPA supplemented with 1% PTC (phosphatase inhibitor cocktail), 1% PIC (phosphatase inhibitor cocktail), and 1% PMSF (phenylmethylsulfonyl fluoride)

3. Methods

3.1 Master mold fabrication – Photo-lithography and machine milling

Photo-lithography

1. On a 3-inch diameter silicon substrate, pattern two layers of photosensitive epoxy with different thicknesses (Fig. 2).
    a. For the first layer (5 μm deep microchannels):
        i. Spin coating SU-8™ 3005 at 4,000 rpm and baking at 95°C for 5 min.
        ii. Expose to UV light through a photomask, followed by a postexposure bake at 95°C for 5 min.
    b. For the second layer (cell culture chambers - 250 μm thick):
        i. Spin coating SU-8™ 3050 at 1,000 rpm, soft-baked first at 65°C for 24 h, then at 95°C for 40 min.
        ii. Expose to UV through a second photomask, followed by a postexposure baked at 65°C for 4 h.
2. Coat the master mold with (tridecafluoro-1,1,2,2-tetrahydro octyl) trichlorosilane to facilitate PDMS release.

Machine milling

1. Design the reservoir mold in SolidWorks software (Fig. 3).
2. Turn on and set up the machine (MDX-50) according to the manufacturer's instructions.
3. Load the SolidWorks file in the SRP Player software.
4. Set and confirm the drill sizes.
5. Before start cutting the sample, set the origin point.

6. Mill it based on the design.
7. When milling is done, click “finish”.
8. Open the cover of the machine and remove the reservoir.

3.2 Fabrication of the device

1. Prepare the PDMS pre-polymer and curing agent mix in a 10:1 mix ratio by weight.
2. Stir with a spoon until the mixture is homogenized and filled with bubbles.
3. Using the appropriate cell chamber or reservoir molds, pour the PDMS mixture:
    a. Fill the reservoir mold to the top till it matches the reservoir marks.
    b. Fill the master mold halfway to the top.
4. Place the molds inside the degassing chamber and run a vacuum pump to remove the bubbles for 30 minutes.
5. Turn off the vacuum pump.
6. Take out the degassed PDMS molds and check for the presence of remaining bubbles.
7. Place the molds inside the oven:
    a. If using high temperature (around 86^{o}C): bake for one hour.
    b. If using low temperature (around 40^{o}C): bake overnight.
8. Take out cured PDMS from the oven and let it cool down.
9. With a razor carefully cut out the PDMS device from the molds. Slowly peel the PDMS out of the master mold.

10. Punch the inlets and outlets according to the design of the device:
    a. 1.5 mm and 4 mm puncher (Fig. 4).
11. Clean the PDMS molds (chambers and reservoirs) with IPA on both sides in a chemical hood.
12. Let it dry inside the hood.
13. Remove the dust with a tape.

Plasma Bonding

14. Using a large glass slide, place the PDMS cell chamber and the cover slip side by side.
15. Place the glass slide into the plasma chamber.
16. Close the door and start the vacuum, waiting to reach 0.3 – 0.4.
17. Turn on the power, open the valve till the gauge read 0.6, and wait 1.5 min.
18. Turn off the power.
19. Release the vacuum until it reached the normal values.
20. Open the door and bond immediately the master mold (side down) to the cover slip.
21. Repeat the process with the reservoir mold and the master mold – coverslip pair.
22. Bond the reservoir mold matching the inlets and outlets of the master mold (Fig. 5).
23. Use PDMS prepared mixture around the reservoir-chip junction.
24. Bake overnight at low temperature.

3.3 Preparing the device before loading

1. Before starting, check if the devices are ready to use: if the plasma bonding is good, the punches align well to the reservoir, and that the slide is not cracked.

From now on, all the steps should be done inside the hood:

2. Pipette 70% ethanol into the chambers and reservoir of each device and let it sit for 15 minutes to sterilize the FMi-OOC.
3. Remove ethanol aspirating with a pipette tip and vacuum.
4. Wash 2 times with PBS.
5. Remove PBS aspirating with a pipette tip and vacuum.
6. Load the AEC chamber with approximately 50 ul of 25% collagen type IV Matrigel.
7. Using a square-shaped piece of PDMS with a single hole punch, aspirate with a pipette tip inserted in the AMC chamber using the vacuum (Fig. 6).

a. Check the formation of “half circles” in the AEC chamber (Fig. 7) which is type IV Matrigel coming through the microchannels.

8. Load AMC chamber with 25% collagen type IV Matrigel.
9. Using a square-shaped piece of PDMS, aspirate with a pipette tip inserted in the CTC chamber using the vacuum:

a. Check the formation of half circles in the AMC chamber.

10. Fill the AEC, AMC, and CTC chamber with 25% collagen type IV Matrigel.

Note: 25% type IV Matrigel mixture cannot fill the reservoirs. remove any extra mixture before the next step.

11. Incubate for at least 4 h at 37^{o}C.

3.4 Loading cells into the devices

1. Remove the collagen manually with a pipette (or with gentle vacuum) from the chambers and check if the chambers are empty.
2. Fill all chambers of the device with serum-free media to keep it hydrated.
3. Passage all cell types (AEC, AMC, CTC, DEC):
    a) Add 2 ml of trypsin to each T75 flask.
    b) Incubate for 4-12 minutes (it will vary according to the cell type).
    c) Neutralize the trypsin with 4 mL of 10% FBS media.
    d) Transfer the content to a 15 mL tube.
    e) Centrifuge for 5 min at 3000 rpm.
    f) Carefully discard the supernatant.
    g) Resuspend the pellet in 250 ul of the appropriate media.
    h) Count cells and prepare one microtube for each type of cell according to Table 1.
4. Remove serum-free media in the device with a pipette.
5. Load the cells slowly and avoid the bubbles (Fig. 8):
    a. Start with the DEC chamber, then CTC, AMC, and finally AEC chamber.
6. Fill up the reservoirs completely with the cell-specific media.

7. Place the devices in a 6-well plate:
    a. Fill the centers of the 6-well plate with 2 mL sterile PBS to limit media evaporation.
8. Incubate for 24 h.

3.5 Propagation experiments

1. Prepare treatments according to the design of the experiment.

Note: if using live bacteria, optimize the number of bacterial cells used in the device.

2. 24 hours after cell loading, check the cells in the microscope to confirm if they are attached, maintain proper morphology, and looking viable (Fig. 9).
3. Remove the media from the reservoirs only.
4. For maternal to fetal propagation:
    a. Control devices: fill reservoirs with cell-specific media according to the gradient: AEC 60 ul/reservoir – AMC 70 ul/reservoir – CTC 70 ul/reservoir – DEC 90ul/reservoir
    b. Treatment: Fill reservoirs from outside (AEC chamber) to inside (DEC chamber), being the treatment the last added:
        i. Add cell-specific media to the AEC, AMC, and CTC chamber following the gradient: AEC 60ul/reservoir – AMC 70 ul/reservoir – CTC 70 ul/reservoir.
        ii. Add treatment to the DEC reservoir: 90 ul.

c. Establish the endpoint according to the experiment design.

i. If collecting the media:

- Prepare and identify microtubes for each cell chamber.
- Collect the media from individual cell reservoirs and chambers carefully using a pipette.
- Change tips between cell types.

ii. If collecting cell lysate:

- Prepare and label microtubes.
- After removing the media, add 40 ul of RIPA buffer in each chamber.
- Place the 6-well plate on ice and incubate for 10 minutes.
- Collect the lysate pipetting in-out 10 times.
- Change tips between chambers.
- Vortex the microtubes for 30 sec.
- Sonicate for 30 sec.
- Place in the –80^{o}C until further analysis.

4. Notes

- The cells used in this device were isolated and characterized according to previous studies and protocols of the group ***(15,16)***.
- Pipetting of cells, media, and any other regents in the chamber, must avoid trapping of bubbles.

- For the CTC and AMC cell loading, both type IV Matrigel and primary collagen need to be added in the respective concentration: 25% of type IV Matrigel for both types of cells and 20% of primary collagen in AMC and 5% in CTC.
- When calculating the amount for each cell chamber according to Table 1, always extrapolate in at least two extra devices.

Acknowledgment

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Tables

Type of cell	Number of cells per chip	Type IV Matrigel (25% stock)	Primary collagen	Total volume per chamber
AEC	85,000	-	-	20.00 ul
AMC	60,000	25% of the total volume	20% of the total volume	35.00 ul
CTC	200,000	25% of the total volume	5% of the total volume	25.00 ul
DEC	45,000	-	-	100.00 ul
Note: This is the amount needed for one device. multiple according to the number of devices used in the experiment design.				

Table 1: FMi-OOC information for loading devices

Figures

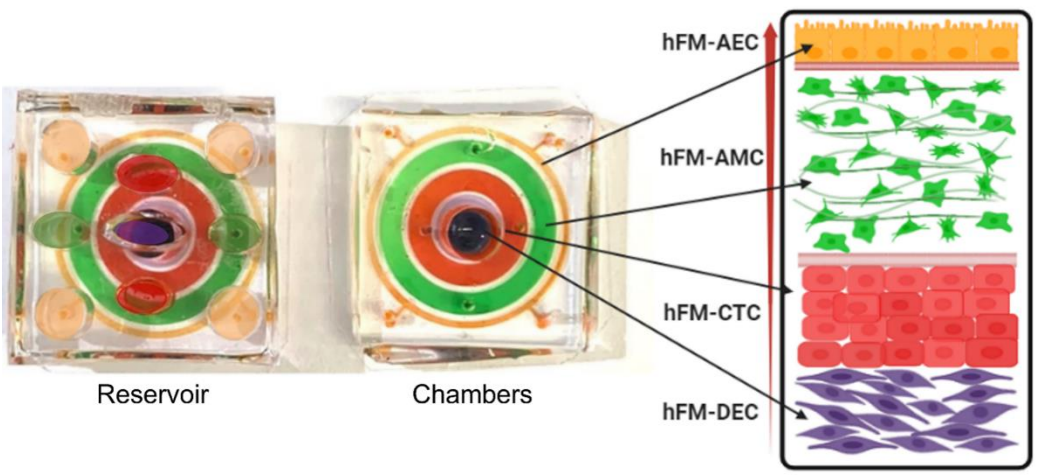

Figure 1. Image and design of Feto maternal interface organ-on-chip. Images of reservoir and chambers of the FMi-OOC, each chamber represented by colors as follows: amnion epithelial (orange), amnion mesenchymal (green), chorion trophoblast (red), and decidual (purple). On the right of the figure, the schematic design with each type of cell that will be added to the device.

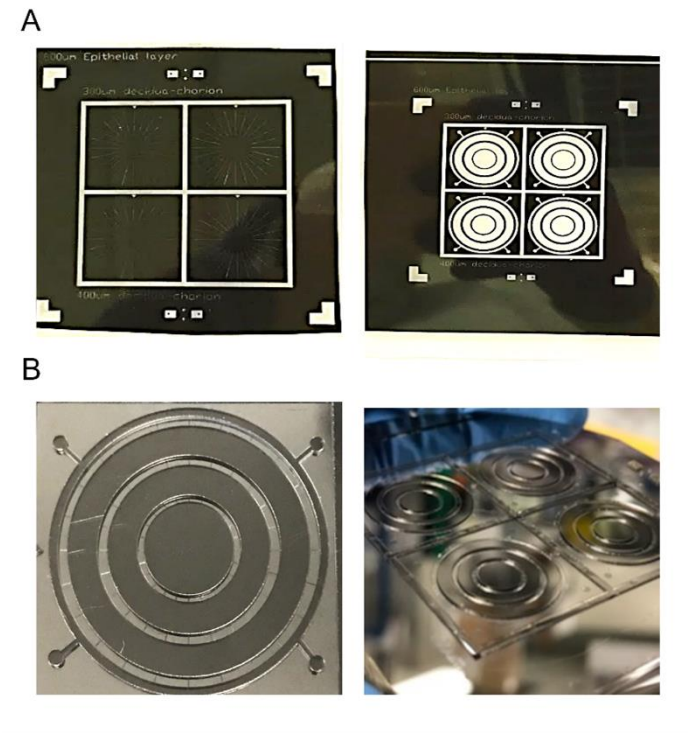

Figure 2. Master mold design and fabrication. A: Graphic design of the master mold. B: Molds ready after photo-lithography process.

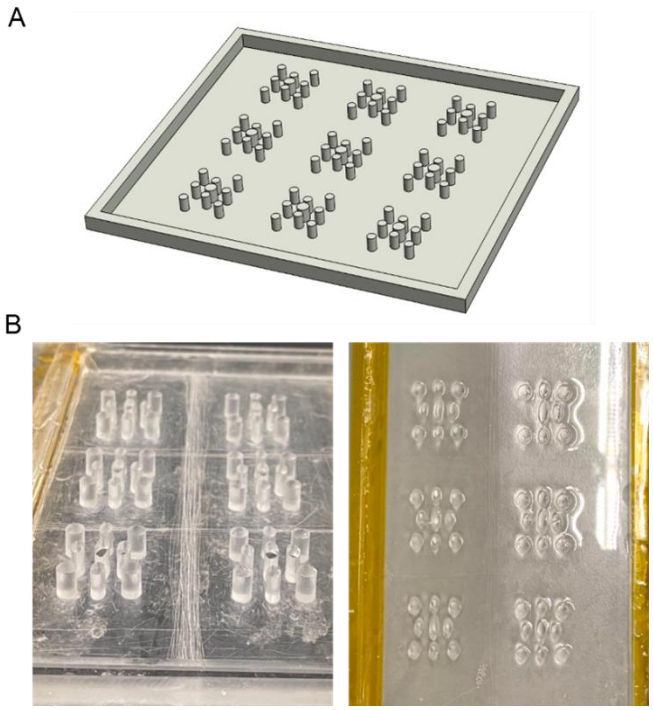

Figure 3. Graphic design and mold printed of the reservoir. A: Graphic design of reservoir mold for the milling machine. B: 3D mold of FMi-OOC reservoir printed (left) and filled with PDMS (right).

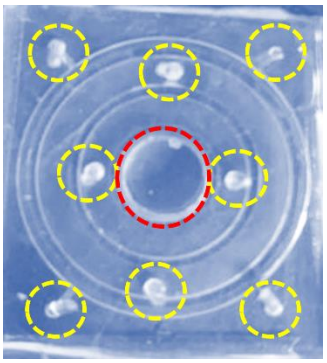

Figure 4. Punches in the inlets and outlets of the master mold. 1.5 mm punches in yellow in the amnion epithelial, amnion mesenchymal, and chorion trophoblast, and 4 mm punch in red in the decidual chamber.

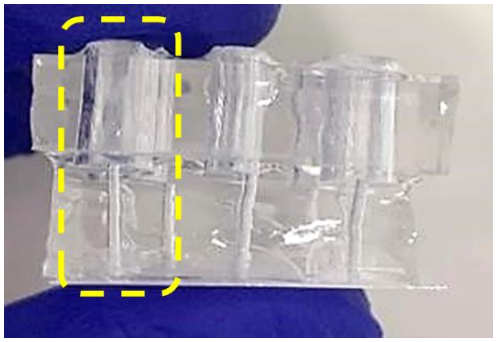

Figure 5. Alignment of master mold and reservoir mold after plasma bonding. The second step of plasma bonding requires the alignment of both molds. On the top is the reservoir mold aligned with the master mold on the bottom.

Figure 6. Type IV Matrigel vacuuming. A square-shaped piece of PDMS with a single hole punch on top of the reservoir mold. A pipette tip (connected to the vacuum) inserted in the punch aligned with the AMC chamber reservoir.

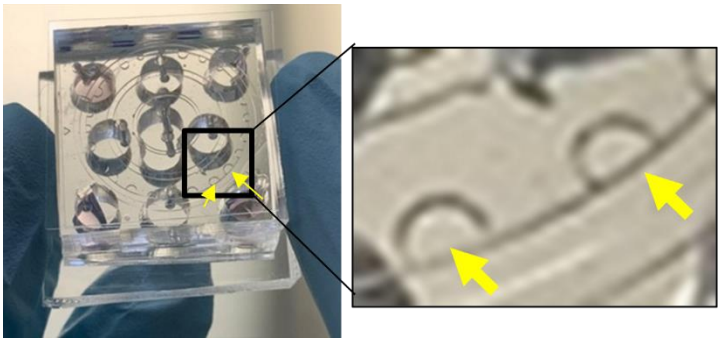

Figure 7. Formation of half bubbles after vacuuming IV Matrigel through the microchannels. Half bubbles formed in the AEC and AMC chamber after adding 25% collagen type IV Matrigel and suctioning using a vacuum.

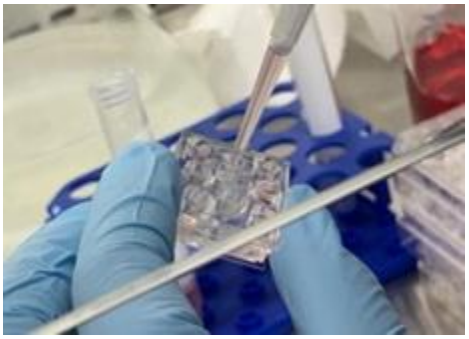

Figure 8. Cell loading of FMi-OOC. After preparing one microtube per type of cell according to Table 1, load each chamber, avoinding bubbles as much as possible.

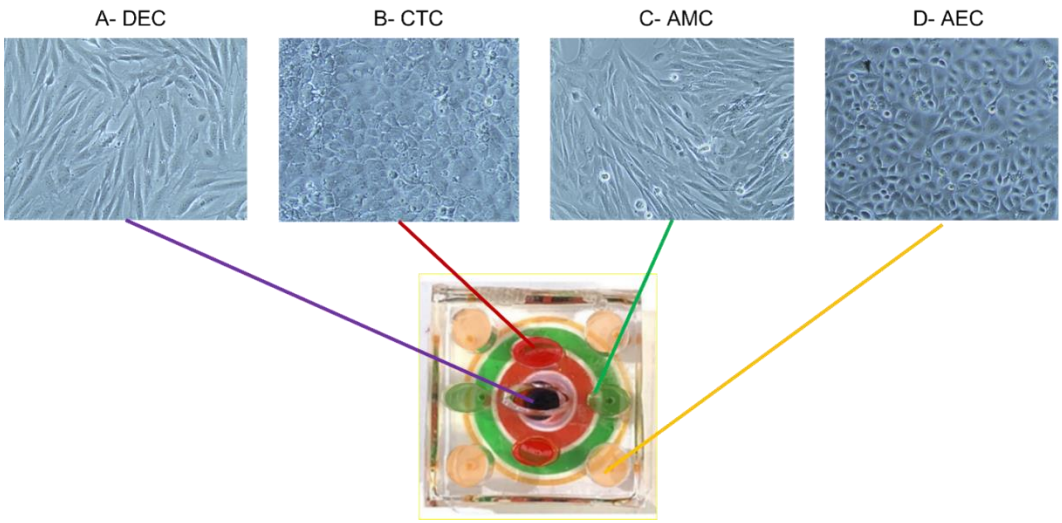

Figure 9. Cell morphology of each type of cell of the FMi-OOC. Morphology of decidual (DEC) (A), chorion trophoblast (CTC) (B), amnion mesenchymal (AMC) (C), amnion epithelial (AEC) (D) cells after 24 hours of loading the FMi-OOC. Bright-field images, 20x magnification**.**

Capítulo III

Artigo Científico 1

Manuscrito publicado no periódico *American Journal of Reproductive Immunology* (Fator de impacto: 3.6).

Modeling an ascending infection by *Ureaplasma parvum* and its cell signaling and inflammatory response at the feto-maternal interface

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Abstract

PROBLEM: Ascending bacterial infection is associated with ~ 40% of spontaneous preterm birth (PTB), and *Ureaplasma* spp. is one of the most common bacteria isolated from the amniotic fluid. Developing novel *in vitro* models that mimic *in vivo* uterine physiology is essential to study microbial pathogenesis. We utilized the feto-maternal interface organ-on-chip (FMi-OOC) device and determined the propagation of *Ureaplasma parvum*, and its impact on cell signaling and inflammation. METHOD OF STUDY: FMi-OOC is a microphysiologic device mimicking fetal membrane/decidua interconnected through microchannels. The impact of resident decidual $CD45^+$ leukocytes was also determined by incorporating them into the decidual chamber in different combinations with *U. parvum*. We tested the propagation of live *U. parvum* from the decidual to the amniochorion membranes (immunocytochemistry and quantitative PCR), determined its impact on cytotoxicity (LDH assay), cell signaling (JESS™ Western Blot), cellular transition (immunostaining for vimentin and cytokeratin), and inflammation (cytokine bead array). RESULTS: *U. parvum* transversed the chorion and reached the amnion epithelium after 72 hours but did not induce cell signaling kinases (p38MAPK and JNK) activation, or cellular transition (epithelial-mesenchymal), regardless of the presence of immune cells. The inflammatory response was limited to the choriodecidual interface and did not promote inflammation in the amnion layer. CONCLUSIONS: Our data suggest that *U. parvum* is poorly immunogenic and does not produce massive inflammatory changes at the feto-maternal interface. We speculate that the presence of *U. parvum* may still compromise the feto-maternal interface making it susceptible to other pathogenic infection.

Keywords: infection; inflammation; fetal membrane; decidua; pregnancy; organ-on-chip; preterm birth.

Introduction

Preterm birth (PTB), the delivery before 37 weeks of gestation, is the leading cause of neonatal mortality and morbidity worldwide. Annually, 15 million babies are born preterm[1], corresponding to 5% to 18% of total pregnancies[2]. Most of the PTBs are spontaneous with unknown etiology; however, ascending infection contributes to 40% of those cases[3]. In most cases, the infection is polymicrobial, and the most common bacteria isolated associated with PTB include genital mycoplasmas, such as *Ureaplasma* spp.[4].

The ambiguity in the association of *Ureaplasma* spp. with adverse pregnancy outcomes, such as preterm premature rupture of membranes (pPROM), preterm labor (PTL), and PTB, was demonstrated recently in a systematic review[5]. Most associations, or lack thereof, can be explained by heterogeneities in studies establishing *Ureaplasma parvum* as a 'causal' pathogen. Various factors contribute to heterogeneities but are not limited to the differences in the timing (gestational age) and type of sampling for microbial detection, microbial load, type of approach used for microbial identification, and whether the infection was detected on the maternal or fetal side[5]. An association between the *Ureaplasma* spp. and inflammation has been shown in the literature; however, these data also remain inconclusive [6-9] for the reasons mentioned above. In summary, the systematic review and other studies reported that *Ureaplasma* spp. alone cannot produce the massive inflammation required to trigger the events leading to preterm labor[5,10-12]. This systematic review's conclusion is consistent with a study on fetal membranes that reported a lack of proinflammatory response by genital mycoplasmas in the absence of a polymicrobial etiology that should also include

Gardnerella vaginalis, *Escherichia coli* [10]. Similar conclusions were made on ascending infection and PTB using animal model studies[13,14]. Conversely, direct administration of *U. parvum* into the amniotic fluid can cause PTB suggesting that feto-maternal interface (FMi) is a major barrier for the propagation of a load of microbe needed to reach the amniotic cavity to cause a fetal inflammatory response needed to drive parturition signals[15]. The cell signaling associated with infection that leads to adverse gestational outcomes has also been studied in recent years. Both p38 MAPK and Jun N-terminal kinase (JNK) are known to be involved in both physiologic and pathologic labor in crucial mechanisms, such as regulation of inflammatory cytokines genes, and apoptosis [16-18]. Group B Streptococcus (GBS) and lipopolysaccharide (LPS) infection were shown to increase the phosphorylated levels of p38 MAPK and JNK [19,20].

Better *in vitro* models are needed to explain the inconclusive role of *Ureaplasma* spp. in PTB and other adverse outcomes after it ascends from the vagina through the cervical barriers. Recent advancements in microphysiologic models, like organ-on-chip (OOC) devices, have helped mimic different intrauterine organs. Here, multiple cell types can be cultured in an interconnected multiwell device mimicking an organ system as seen *in utero* [21-23]. Our group has established several models of OOC recently[14,24-26], and the fetal membrane feto-maternal interface organ-on-chip (FMi-OOC) [27] recreated both maternal (i.e., decidua) and fetal sides (i.e., amniochorion). This model has been used previously to test pathogenic changes caused by infectious agents (Lipopolysaccharides [LPS]) [27], environmental chemicals at the FMi [28], and the pharmacokinetics of

drugs [26]. In addition, an OOC device consisting of vaginal-cervical-decidual regions has reported *U. parvum* infection in the lower genital tract [14,29], showing that these models can be used to study infection-associated pathophysiological processes.

Current *in vitro* 2D and *in vivo* animal models have limitations in generating supportive evidence for the hypothesis that *Ureaplasma* is mechanistically associated with producing inflammation required at the FMi tissues. To better understand the pathogenesis of the bacteria, it is essential to know the role of immune cells in ascending infection since pregnancy shows a balanced immune response at the FMi. *S*tudies have shown that $CD45^+$ leukocytes (e.g., ~66 % T cells, ~13% NK cells, ~4% macrophages, ~4% B cells, ~1.2% neutrophils) [30] are increased in fetal membranes from at-term labor compared to a term not in labor subjects [31]. The objective of this study is to recreate the FMi *in vitro* using an OOC device to test the pathogenic properties of *Ureaplasma* and its ability to cause FMi cell inflammation if it propagates from decidua to the fetal membranes. Using the FMi-OOC that contained decidual and three types of fetal membrane cells (chorion trophoblast, amnion mesenchyme, and epithelium), this study tested: **1)** propagation of *U. parvum* from decidual cells to the fetal membrane cells**, 2)** the impact of *U. parvum* infection on cell signaling indicative of pathophysiology resulting in inflammation, and **3)** the influence of decidual $CD45^+$ cells in the immune response at the FMi in contributing to *U. parvum* pathology.

Methods

1. Cell culture

1.1.Decidual and fetal membrane cells

The collection of the placenta to be used in this study as a discarded human specimen, was approved by the Institutional Review Board (IRB) at the University of Texas Medical Branch at Galveston, TX (IRB approval number IRB16.0058, January 2020). Primary decidual and fetal membrane cells were isolated from elective term Cesarean section, not in labor placenta, and immortalized as reported previously [32]. These cell lines were obtained from different fetal membrane samples included in our study. Human Fetal Membrane – Decidual cells hFM-DEC (DEC) and hFM-AMC - Human amnion mesenchymal cells (AMC) were cultured in Dulbecco's Modified Eagle Media (DMEM)/F12 (Mediatech Inc., USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Mediatech Inc., USA), and 1% amphotericin B (Sigma-Aldrich, USA). hFM-CTC - Human chorion trophoblast cells (CTC) were cultured in DMEM/F12 supplemented with 0.20% FBS, 0.1 mM β-mercaptoethanol, 0.5% penicillin/streptomycin, 0.3% bovine serum albumin (BSA), 1× ITS-X, 2 μM CHIR99021, 0.05 μM A83-01, 1.5 μg/mL L-ascorbic acid, 50 ng/mL−1 epithelial growth factor, 0.08 mM valproic acid (VPA), and 1× Y27632 (Rock inhibitor). hFM-AEC - Human amnion epithelial cells (AEC) were cultured in Keratinocyte serum-free medium (KSFM) supplemented with bovine pituitary extract (30 μg mL), epidermal growth factor (0.1 ng mL), CaCl2 (0.4

168 mM), and primocin (0.5 mg mL). All cell types were grown at 37 °C and 5%
169 CO_2.

170 **1.1. CD45$^+$ leukocyte cells**

171 CD45$^+$ cells were isolated from the decidua to incorporate in our model. We
172 collected placenta from elective term Cesarean section, not in labor, as described
173 in previous studies [33,34]. Decidual tissue was removed from the chorion and
174 digested with DNAse (Sigma-Aldrich, USA) and collagenase (Sigma-Aldrich,
175 USA). After digestion, red blood cells were lysed, washed, and CD45$^+$ cells were
176 isolated using Ficoll plaque density gradient (Cytiva, USA). CD45$^+$ cells were
177 maintained in RPMI-1640 (Gibco, USA) media supplemented with 10% FBS and
178 5% penicillin/streptomycin (Mediatech Inc., USA). The purity of the CD45$^+$ cells
179 isolated from decidua was confirmed by flow cytometry and
180 immunocytochemistry using an Anti-CD45 antibody (dilution 1:200, ab30470,
181 Abcam, Inc.) (data not shown).

182 **2. Fetal Maternal interface organ-on-chip (FMi-OOC)**

183 **2.1.Device design**

184 The FMi-OOC devices were designed and manufactured by our laboratory as
185 previously described [27]. The device has four chambers made of
186 Poly(dimethylsiloxane) (PDMS) which are connected through 24 microchannels.
187 Each chamber was designed for one of the four cell types that compose the FMi:
188 the center chamber (1) contains maternal decidual cells (purple), chamber 2

contains CTC (blue), followed by chamber 3, which contains AMC (green), and finally, the outer chamber (4) has AEC (orange) (**Figure 1**). A reservoir block also made of PDMS is aligned on top of the cell culture chamber, matching the outlets and inlets. The devices were sterilized with 70% ethanol and washed with phosphate-buffered saline (PBS) before use. Microchannels between the AEC/AMC and AMC/CTC layers were filled with type IV basement membrane collagen Matrigel (Corning Matrigel , 1:25 in serum-free media) and incubated at 37°C with 5% CO_2 for 4 hours to mimic the basement membranes *in utero*. All cell chambers were rinsed with serum-free media , and the devices were loaded as follows: 45,000 DEC in chamber 1; 200,000 CTCs + 5% primary collagen + 25% Matrigel in chamber 2; 60,000 AMCs + 20% primary collagen + 25% Matrigel for chamber 3; and 100,000 AECs for chamber 4. Finally, the FMi-OOCs were incubated at 37°C with 5% CO_2 for 24 hours before treatments.

2.2 ***U. parvum*** **culture**

U. parvum (ATCC® 700970™) was obtained from the American Tissue Culture Collection (ATCC). *U. parvum* was propagated in UMCHs medium [35]: Mycoplasma broth base (Becton, Dickinson and Co., Baltimore, MD) 1.47% (wt/vol) supplemented with horse serum (Biowhittaker, USA) 20% (vol/vol), yeast extract (Becton, Dickinson and Co., USA) 2.5% (wt/vol), L-cysteine hydrochloride 0.01% (wt/vol), urea 0.04% (wt/vol), phenol red 0.001% (wt/vol), and penicillin G 1000 U/ml. *U. parvum* cultures were incubated for 12 – 14 hours, and the amounts of Ureaplasma DNA were verified using a genesig Std Real-time

PCR detection kit (Z- Path- *U. parvum*- std, American Research Products Inc., USA) and they amounted to 1.54 × 10E07 copy numbers/ml.

3. Experiment setup and assays

3.1. $CD45^+$ cells

After isolation, 1x10E06 $CD45^+$ cells were seeded in a 6-well plate with the appropriate media and treated with 1x10E05 DNA copies/mL of *U. parvum*. $CD45^+$ cell culture and media without any cells were used as controls. After 24 hours, the supernatant was collected, centrifuged to remove cell debris, and stored at -80^{o}C. The supernatant from $CD45^+$ cells treated with *U. parvum* was included in subsequent models to recreate the contributions of immune cells in the decidual region in our FMi-OOC models. Media from untreated cells and blank media (no cells) were included as a control to confirm the contributions of decidual immune cells during an infectious process of the FMI. To note, $CD45^+$ cells were not included in our FMi-OOCs.

3.2. FMi-OOC experiment

The FMi-OOC devices were seeded with specific cell types. After 24 hours, different types of treatments (**Table 1**) (n=5) were added to the decidual chamber: (1) regular DEC media as a negative control, mimicking a scenario with no infection in the FMi(CTL); (2) live *U. parvum* to determine the ascending infection (UP^+); (3) supernatant from decidual $CD45^+$ immune cells along with live *U. parvum.* This combination tested decidual response to live bacteria when

immune cell secreted factors are present; (4) supernatant of $CD45^+$ cells treated with *U. parvum.* This combination tested whether metabolites from immune cells exposed to *U. parvum* would cause changes at the FMi-OOC ($CD45^+$), and (5) supernatant of CD45 previously treated with *U. parvum* and live infection of *U. parvum* to test if live infection of *U. parvum* would be affected by immune cells with previous contact with the microorganism ($CD45^+UP^+$). For the propagation experiment, *U. parvum* at a concentration of 1x10E05 DNA copies/mL was added in the decidua chamber. Media from all the chambers was collected at 24 hours, 48 hours, and 72 hours for the rt-PCR detection of *U. parvum (*Z- Path- *U. parvum*- std, American Research Products Inc., USA). The cells on the devices were fixed with ethanol and used for immunocytochemical staining of multiple banded antigen (MBA) (#MA5-17010, Invitrogen), a virulence factor of *U. parvum*.

The treatments mentioned above **(Table 1)** were added to the decidua chamber to check for cell morphology, inflammatory cytokine production, and protein analysis of cell signaling markers. After 72 hours, media was collected, and cells were either lysed or fixed from all chambers and stored at -80^o for further analysis (Supplementary Figure 1).

4. Immunocytochemistry

Immunocytochemical staining for MBA (dilution 1:200, #MA5-17010, Invitrogen) in the FMi-OOC was performed 24 hours, 48 hours, and 72 hours post-infection with *U. parvum* to confirm propagation. To determine cell morphology and cellular transition

status (i.e., epithelial to mesenchymal transition [EMT] or mesenchymal to epithelial transition [MET]) vimentin (dilution 1:300, #ab92547; Abcam, Inc.) and cytokeratin 18 (dilution 1:200, # ab668, Abcam, Inc.) were used for the staining of DEC/AMC and CTC/AEC, respectively.

After each time point, cells were fixed with 70% ethanol overnight, washed with PBS, and blocked with 3% bovine serum albumin in PBS before incubation with primary antibodies overnight at 4°C. After washing with PBS , slides were incubated with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Life Technologies, Carlsbad, CA) and diluted 1:1000 in PBS for 1 hour in the dark. Slides were washed with PBS, treated with NucBlue Fixed Cell Stains ReadyProbes Reagent (R37606; Thermo Fisher Scientific) for 5 minutes, and then mounted using Mowiol 4 to 88 mounting medium (475904–100GM-M; Sigma-Aldrich, Inc.).

5. Automated Western immunoblotting – JESS™

The lysates of each chamber in the FMi-OOC were collected as follows: 40ul of the combination of radioimmunoprecipitation assay (RIPA) supplemented with 1% PTC (phosphatase inhibitor cocktail), 1% PIC (phosphatase inhibitor cocktail), and 1% PMSF (phenylmethylsulfonyl fluoride) was added in each chamber, collected after 10 minutes of incubation on ice, vortexed, sonicated for 30 seconds, and stored at -80°C. The protein concentration of the samples was calculated using Bicinchoninic Acid Method (BCA, Pierce, Rockford) after centrifuging at 12,000 RPM at 4°C. for 20 minutes.

Protein levels of p38 mitogen-activated protein kinase (MAPK) total (dilution 1:50, #9212, Cell Signaling Technology), phospho-p38 MAPK (dilution 1:50, #9211, Cell Signaling Technology, Danvers, MA), and phospho- Jun N-terminal kinase (JNK) (dilution 1:50, #9251, Cell Signaling Technology, Danvers, MA) were analyzed using the capillary-based instrument Simple Western JESS™ (Protein Simple). After optimization of the dilution of the antibodies and the sample concentration (0.2ug/ul), the assays were performed following the manufacturer's protocol. Samples were diluted in 0.1x sample buffer (#042-195), and Fluorescent Master Mix (#PS-ST01EZ-8) was added for denaturation for 5 min at 95°C. Anti-rabbit (#042-206) or Anti-mouse secondary (#042-205) horseradish peroxidase (HRP)-labeled antibodies were used according to the species of the primary antibody. Replex (ProteinSimple, RP-001) module and β-actin (dilution 1:25, #ab8226, Abcam, Cambridge, MA) were used to normalize each plate. Data were analyzed using Compass for Simple Western software v4.0.0. The resulting area of each specific molecular weight peak was divided by either the β-actin value in the same capillary or the total protein value of the respective antibody for normalization.

6. LDH assay

Lactate dehydrogenase (LDH) cytotoxicity detection kit (#11644793001, Roche Diagnostics, IN, USA) was used according to the manufacturer's instructions to check the cytotoxic effect of the treatments in the cell types. Approximately 10µl media from each chamber was collected after 72 hours, and

90ul of LDH solution was added to each well in a 96-well plate. Specific cell culture media of each cell type was used as the negative control, and cell supernatant treated with the mixture of 12% of Triton X-100 in 1X PBS was used as a positive control. After incubation at room temperature for 20 minutes in the dark, absorbance was measured at 450nm using a microplate reader.

7. Enzyme-linked immunosorbent assay (ELISA)

After 72 hours, supernatants were collected of all cell types. Multiplex assays were performed for tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF) (#HCYTA-60K, Millipore, Merck), and competitive ELISA kit for progesterone (#EIAP4C21, Invitrogen, Thermo Fisher)**.** The assays were performed according to the manufacturer's instructions. Standard curves were developed from known quantities of recombinant proteins provided. Sample concentrations were determined by relating the fluorescence values obtained to the standard curve by linear regression analysis.

8. Statistical analysis

Data were analyzed using GraphPad Prism software version 8 (GraphPad Software, San Diego, CA). To check the normality, the Shapiro-Wilk test was performed. Parametric data were analyzed by t-test or one-way ANOVA followed by Tukey comparison post hoc. The Kruskal-Wallis test, followed by Dunn's multiple comparisons or Mann-Whitney U test, was used for non-parametric data. Statistical significance differences are indicated by $p < 0.05$.

Results

1. Cytotoxic effects of *U. parvum* in the FMi cells

Prior to testing the pathogenic properties of *U. parvum*, cytotoxicity (if any), induced by *U. parvum* colonization of the cells of the FMi-OOC were tested. LDH assay was conducted with the media collected from all device chambers for this. Although higher in $CD45^+$ and $CD45^+UP^+$ treated, the cytotoxicity < 15% in AECs is in the acceptable biological range expected in these experiments (**Figure 2**). None of the other treatments or cells showed cytotoxicity.

2. Propagation *of U. parvum* across the FMi

We recreated the fetal membrane-decidual interface using a microphysiologic device to test the pathogenic properties of an ascending *U. parvum*. This model is expected to mimic *in utero* environment and, most importantly, maintain intercellular interaction between cellular and collagen layers of the maternal-fetal interface organ system. To determine if *U. parvum* can transverse the FMi-OOC, mimicking *in utero* ascending infection, 1x10E05 DNA copies/mL of *U. parvum* were added to the DEC chamber after establishing an adherent cell culture in each chamber. Quantitative PCR detected *U. parvum* only in the DEC chamber after 24 hours (55,118 ± 7,981 DNA copies); after 48 hours, both CTC and AMC chambers tested positive for *U. parvum*; and after 72 hours, *U. parvum* was detected in all chambers, including the AEC chamber (**Figure 3**) which was the farthest from decidua. To confirm the presence of bacterial antigen (and not just DNA), immunostaining for MBA, a virulence factor of *Ureaplasma* spp., was

performed. Figure 3B (yellow circle) shows that MBA localization in cells in all chambers confirmed PCR results. In summary, our data showed that *U. parvum* colonized all layers of the FMi-OOC after 72 hours. Hence, this time point was chosen for the rest of the experiments to determine the pathogenic effects of *U. parvum* in all the FMi-OOC cells.

3. Cell signaling mechanisms activated by *U. parvum* infection in the FMi cells.

Different treatments were established in this study to mimic different scenarios **(Table 1)**: negative control (CTL), live infection with *U. parvum* (UP^+), and decidual immune cells in multiple combinations with *U. parvum.* To determine cell signaling associated with *U. parvum* infection, stress-activated signaling kinases p38 MAPK and JNK levels were measured in the cells collected from the FMi-OOC by multiplexed western immunoblotting using JESS™. p38 MAPK activation via phosphorylation is a well-documented process critical for the induction of preterm and term labor onset [16,17]. This stress-signaling kinase is a major contributor to fetal membrane dysfunction through various cellular derangements [36,37]. This study determined the phosphorylated p38 MAPK (P-p38 MAPK -Thr180/Tyr182) and total p38 MAPK levels after the treatments. Although the total p38 MAPK in CTC (p=0.037) and AMC (p=0.012) treated with primed immune cells ($CD45^+$) was increased compared to the control, the active P-p38 MAPK/total p38 MAPK ratio was not altered by the different *U. parvum* treatments compared to control **(Figure 4)**. These findings suggest that *U.*

parvum, irrespective of the presence of decidual immune cells, did not activate p38 MAPK. JNK is in the family of MAPK related to cellular senescence, apoptosis, and oxidative stress-induced inflammation, all critical cell fates and processes within fetal membrane cells during pathologic preterm or physiologic term labor [18,38]. Compared to the control, there was no difference in P-JNK (threonine 183/tyrosine 185 residues) levels in the other treatments, regardless of the type of cells. (**Figure 5**). These findings showed that *U. parvum* infection did not affect the P-JNK, suggesting no apoptosis or JNK-derived inflammation induction. In summary, two MAPKs, generally associated with cellular stress and inflammatory signaling, are not impacted by *U. parvum* infection of the FMi cells.

4. *U. parvum* infection does not affect cell morphology or cellular transition status in the FMi cells.

As infectious stimulants (i.e., LPS) and infectious inflammation (i.e., TNFα) have been shown to induce changes in fetal membrane cell morphology and cause cellular transition (i.e., induction of EMT or MET) in amnion cells, we investigated the potential for *U. parvum* to induce similar cellular level changes by immunocytochemistry for cytoskeletal markers (i.e., vimentin [mesenchymal] and cytokeratin-18 [epithelial]) after treatments. **Figure 6** shows that regardless of treatment, DECs maintained their elongated mesenchymal morphology and vimentin dominance, CTCs remained cuboidal and cytokeratin-18 dominated cell population, AMCs expressed vimentin and had fibroblastoid morphology, while AECs expressed cytokeratin-18 and a coble stone cuboidal cell shape. These data

suggest that infection of *U. parvum* with or without immune cell components neither altered the cell morphology nor induced cellular transitions of the FMi cells.

5. Inflammatory effects of *U. parvum* in the FMi cells

After confirming the propagation of *U. parvum* across the FMi and verifying the absence of cellular or cytotoxic effects, we determined the impact of *U. parvum* and immune cells in the inflammatory response. The media from all the individual cell layers of the FMi-OOC were used to measure the concentrations of TNFα, IL-1β, IL-6, IL-8, IL-10, and GM-CSF (**Figure 7**). Except for IL-1β (data not shown), all other cytokines tested showed measurable levels in all cell types. In summary, *U. parvum* infection alone did not increase inflammatory cytokine production in FMi cells. However, live *U. parvum* infection of the DEC in the presence of immune cells in DECs significantly increased both pro- and anti-inflammatory cytokines in DEC and CTC. To note, DEC contained supernatant from immune cells either primed or unprimed by *U. parvum*.

The presence of *U. parvum* alone (Group 2 - UP^+) increased the levels of IL-8 (p=0.018) in DEC cells. Group 3 ($CD45^-UP^+$) had significantly higher levels of TNFα (p=0.033), IL-6 (p=0.018), and IL-10 (p=0.014) in the DEC chamber, and higher levels of IL-6 (p=0.006) and GM-CSF (p=0.002) in the CTC chamber compared to the control treatment. In AMC cells, this treatment increased IL-10 production (p=0.017). The treatment with immune cells previously treated with *U. parvum* (Group 4 - $CD45^+$) only increased levels of GM-CSF (p=0.005) in

DEC cells and levels of IL-8 (p=0.035) in CTC cells. Finally, Group 5 ($CD45^{+}UP^{+}$) significantly increased GM-CSF (p=0.027) in DEC cells, TNFα (p<0.001) , and IL-8 (p=0.026) in CTC cells compared to the control group after 72 hours. In addition, progesterone assay (P4) in CTC cells did not show a significant difference after the treatments of the study (**Supplementary Figure 2**). The inflammatory response observed in the DEC and CTC was not observed in the AMC and AEC, suggesting that maternal *U. parvum* infection induced a mild localized inflammatory response **(Supplementary Table 1)**. Overall, *U. parvum* infection did not promote inflammation in the amnion layer but, interestingly, promoted an anti-inflammatory response (i.e., increased IL-10 production), suggesting a compensatory effect seen at the choriodecidua interface.

These findings showed that although *U. parvum* can ascend from the maternal to the fetal side, it did not cause cytotoxicity, cellular transitions, signaling kinase activation capable of activating inflammation, and a massive inflammatory response. The presence of *U. parvum*-treated decidual immune cell components was insufficient to mount an inflammatory response. These data support the systematic review and prior reports using fetal membrane explants and organ-on-a-chip devices [5,10,14].

Discussion

Studies about *U. parvum* have reported that they have poor immunogenicity, does not trigger a massive inflammatory response in human samples, and does not promote PTB in mouse models, which could be explained by this specie being a

commensal of rodents, resulting in an immune response that may be different compared to humans [5,11,14]. Using the FMi-OOC model, we tested the ascending infection of *U. parvum* and its pathogenic properties on the FMi . Our main findings include : 1) *U. parvum* propagated from the maternal decidua to the fetal amnion epithelial side within 72 hours; 2) *U. parvum* did not exhibit cytotoxicity in any cell types; 3) *U. parvum* infection was not associated with the activation of p38 MAPK and JNK pathways; 4) *U. parvum,* either alone or combined with immune cells promoted an increase in pro- and anti-inflammatory cytokine levels in the choriodecidual layer, but the amnion layer was refractory to the presence of *U. parvum*. In summary, *U. parvum* can ascend and propagate through the feto-maternal interface; however, it did not result in cell signaling or cellular derangement to cause a massive inflammatory response. Our model is unique in that we included decidual immune cells and tested its contribution during *U. parvum* infection of the FMi. Lack of any significant immune response, supported the hypothesis that *U. parvum* is not a powerful immunogenic bacterium at the FMi.

U. parvum effects in response to ascending infection and its role in preterm parturition were based on *in vitro* 2D models that use a single cell culture or animal studies [11,39-42]. Differences between human and animal models of pregnancy and PTB have raised concerns regarding the use of data. Specifically, rodent models are often used for PTB and infection model studies. The chorion trophoblast layer is rather obscure at the mouse FMi, compared to the human chorion layer. In humans, multilayered chorion trophoblast forms a barrier and

453 shows constitutive expression of HLA-G. Chorion also produces large quantities
454 of progesterone, an anti-inflammatory factor, and expresses key membrane
455 progesterone receptors (PGRMC1 and 2) [43] to create a major barrier function. In
456 addition, *in vitro* 2D cultures have some limitations as they lack cell-cell
457 interactions, especially when studying an interface with different types of cells
458 [40,44,45] from two physiologically separate individuals (mother and the fetus). The
459 FMi-OOC overcomes these challenges and provide a better scenario to study this
460 interface. *U. parvum* has already been studied in another type of OOC. Tantengco
461 et al. developed the vagina-cervix-decidua OOC and reported the ascending
462 infection of *U. parvum* from the vagina to the decidua, showing that *U. parvum*
463 can ascend, but not cause either cell death or inflammation [14]. Our results showed
464 that FMi-OOC is a good model for studying ascending infection of *U. parvum*
465 past the decidua. Together, these findings propose a better model to study
466 ascending infection that mimics the propagation of infectious agents *in vivo*. As
467 reported by Romero et al. almost three decades ago, ascending infection is
468 characterized by vaginitis, deciduitis, chorionitis, and amnionitis, leads to
469 intraamniotic infection and inflammation predisposing to PTB [46,47]. The two OOC
470 devices can recreate the path of infection and inflammation. In summary, we
471 demonstrated that *Ureaplasma* can ascend from the vagina to the amnion layer
472 and interact with decidual immune cells, yet their propagation is not associated
473 with an inflammatory response. It is well known that decidual immune cells have
474 an important role in maintaining the maternal-fetal immunotolerance throughout
475 pregnancy[48,49]. Changes in decidual immune cells have been associated with

adverse gestational outcomes, such as preeclampsia[50]. Localized inflammation by specific cell types is observed, but overall, it is balanced**.** Cell death and other inflammation-producing cell fate-associated signaling molecules were also not changed, suggesting that the *U. parvum* infection alone is insufficient to elicit a massive inflammation required to trigger labor-associated changes. We have already reported a disease model with LPS using this model where propagation of LPS was associated with massive inflammation mimicking *Escherichia coli*[27]. We did not use LPS in this model as we have already reported that and physiologically validated using animal models [14,27]. These data provided immense credence to this model system.

The localized inflammation in specific cell types is likely due to the presence of an antigen and stress induced by the *U. parvum* resulting in activation of stress kinases such as p38 MAPK or JNK. Previous studies showed that p38 MAPK could be activated by cigarette smoke extract, an oxidative stress inducer, and lead to cellular senescence [51], though this is not observed in this model. We also studied JNK, which is vital in apoptotic signaling, though *U. parvum* did not cause significant activation of JNK regardless of the type of cell. This suggests that *U. parvum* does not induce JNK-associated changes in fetal membrane cells. We speculate that *U. parvum* infection, inflammation, and other pathogenic consequences are location and load dependent. In endometrium or even fetal lung and brain, it is likely to cause an inflammatory response and associated pathologies [52-54]; however, colonization of the amniochorionic membrane or amniotic fluid by *U. parvum* alone is unlikely to induce labor. Location shifts

from amniotic fluid to fetal tissues can be detrimental to the fetal organs, as often observed [55]. Infection of the amniotic cavity and the fetus with *Ureaplasma* spp. can promote bronchopulmonary dysplasia, intraventricular hemorrhage, and necrotizing enterocolitis [55,56].

This is the first time we showed *U. parvum* colonization and its pathologic effects in fetal membrane cells using an FMi-OOC platform. However, this study had some limitations, including 1) not mimicking long term infection and migration of leukocytes from maternal and fetal sides and analyzing a short time infection (72 hours), 2) using a single dose of *U. parvum* as treatment, though the dose chosen was based on the amount of *U. parvum* DNA copies found in the amniotic fluid [57], and 3) lack of $CD45^+$ cells co-culture that did not allow for visualized of immune cell migration. Further studies will be conducted to address the short comings of this study. *U. parvum* alone may not be sufficient to cause PTB, but a combination of other pathogenic microbes can exaggerate the immune response leading to PTB.

Conclusion

We conclude that microphysiologic models can recreate *in utero* environments like choriodecidual interfaces to study pathophysiologic changes associated with PTB. We report that the load of *U. parvum* reported being associated with PTB is incapable of an inflammatory response required to drive PTB pathways. Our data support the systematic review based on clinical studies [5,12] and animal model studies reported by Pavlidis, Tantengco et al. [11,12,14].

However, we speculate that the presence of *U. parvum* in the FMi may still compromise the integrity of the structure, making the FMi more susceptible to infection with other pathogenic species that could trigger pPROM, PTL, and PTB.

Abbreviations List

AEC – hFM-AEC - Human amnion epithelial cells

AMC – hFM-AMC - Human amnion mesenchymal cells

ATCC – American Tissue Culture Collection

BCA – Bicinchoninic Acid Method

BSA – Bovine serum albumin

CTC – hFM-CTC - Human chorion trophoblast cells

DEC – hFM-DEC - Decidual cells

DMEM – Dulbecco's Modified Eagle Media

EMT – Epithelial-to-mesenchymal transition

FBS – Fetal bovine serum

FMi – Feto-maternal interface

FMi-OOC – Feto-maternal interface organ-on-chip

GM-CSF – Granulocyte-macrophage colony-stimulating factor

HRP – horseradish peroxidase

ICC – Immunocytochemistry

IL – Interleukin

JNK – Jun N-terminal kinase

KSFM – Keratinocyte serum-free medium

LDH – Lactate dehydrogenase

MAPK – Mitogen-activated protein kinase

MBA – Multiple banded antigen

MET – Mesenchymal-to-epithelial transition

OOC – Organ-on-chip

P4 – Progesterone assay

PBS – Phosphate-buffered saline

PDMS – Poly(dimethylsiloxane)

PIC - Phenylmethylsulfonyl fluoride

pPROM – Preterm premature rupture of membranes

PTB – Preterm birth

PTC – Phosphatase inhibitor cocktail

PTL – Preterm labor

RIPA – Radioimmunoprecipitation assay

TNFα – Tumor necrosis factor alpha

VPA – Valproic acid

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777 **Tables**

778 **Table 1. Different types of treatments in the decidua chamber of the FMi-**
779 **OOC.**

Groups	Treatment	Specifications	DEC media	*U. parvum*	$CD45^+$ cells supernatant	$CD45^+$ cells *U. parvum* treated supernatant
Group 1	CTL	Regular DEC media (control)	+	-	-	-
Group 2	UP^+	Live *U. parvum* at a concentration of 1x10E05 DNA copies/mL	+	+	-	-
Group 3	$CD45^-UP^+$	Supernatant of $CD45^+$ cells only and live *U. parvum*	+	+	+	-
Group 4	$CD45^+$	Supernatant of CD45 cells treated with *U. parvum*	+	-	-	+
Group 5	$CD45^+UP^+$	Supernatant of CD45 cells treated with *U. parvum* and live *U. parvum*	+	+	-	+

780

Figures

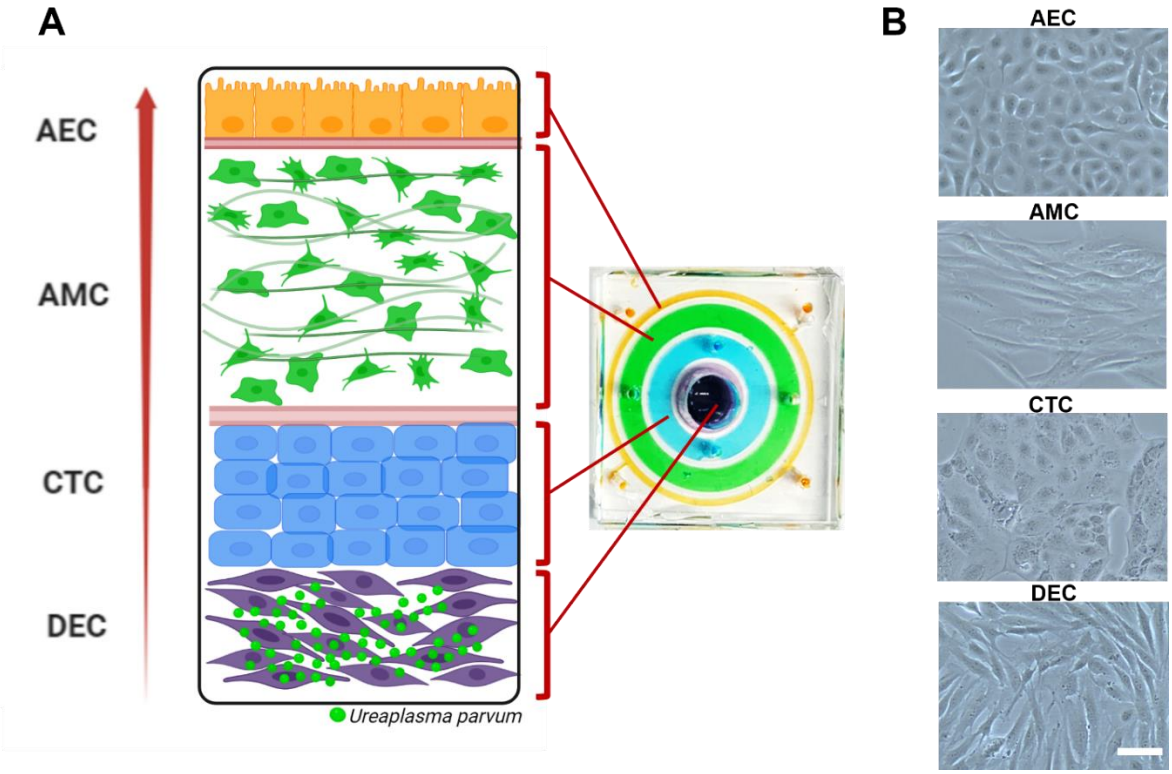


Figure 1. Schematic design of the FMi-OOC and experimental design. **A)** The device is composed of four circular chambers connected by microchannels. The chambers correspond as follows: decidual chamber (purple), chorion trophoblast chamber (blue), amnion mesenchymal chamber (green), and amnion epithelial chamber (orange). By adding *U. parvum* in the decidua chamber, it will mimic the ascending infection model. The schematic design was created using BioRender. B) Bright field images of each type of cell in each chamber of the device – 20x magnification. Scale bar: 25 µm.

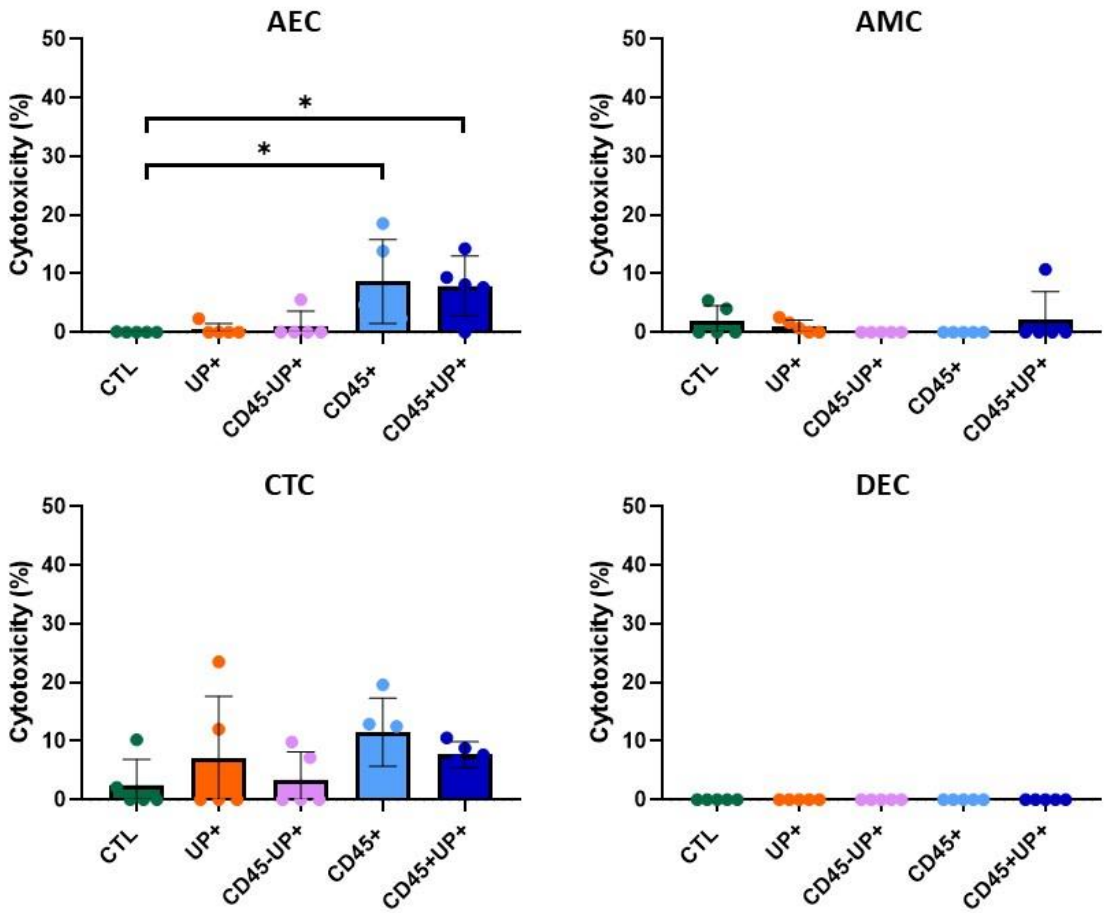


Figure 2. Cytotoxic effects of *U. parvum* infection in the FMi-OOC.
LDH assay was performed with the media of each type of cell in every treatment after 72 hours. Data are presented as mean±SEM. N=5. *, $p < 0.05$. **, <0.01. ***,<0.001

A

DNA copies	DEC	CTC	AMC	AEC
24 h	55,118 ± 7,981	0.00	0.00	0.00
48 h	14,113 ± 6,607	15,663 ± 2,042	7,130 ± 960.7	0.00
72 h	10,380 ± 1,189	23,760 ± 2,136	14,757 ± 613.7	10,717 ± 490.8

N=3, Cq values above 35: negative for *U. parvum.* Values of DNA copies was calculated based on the standard curve of the run. If Cq > 35, the amount of DNA was not calculated.

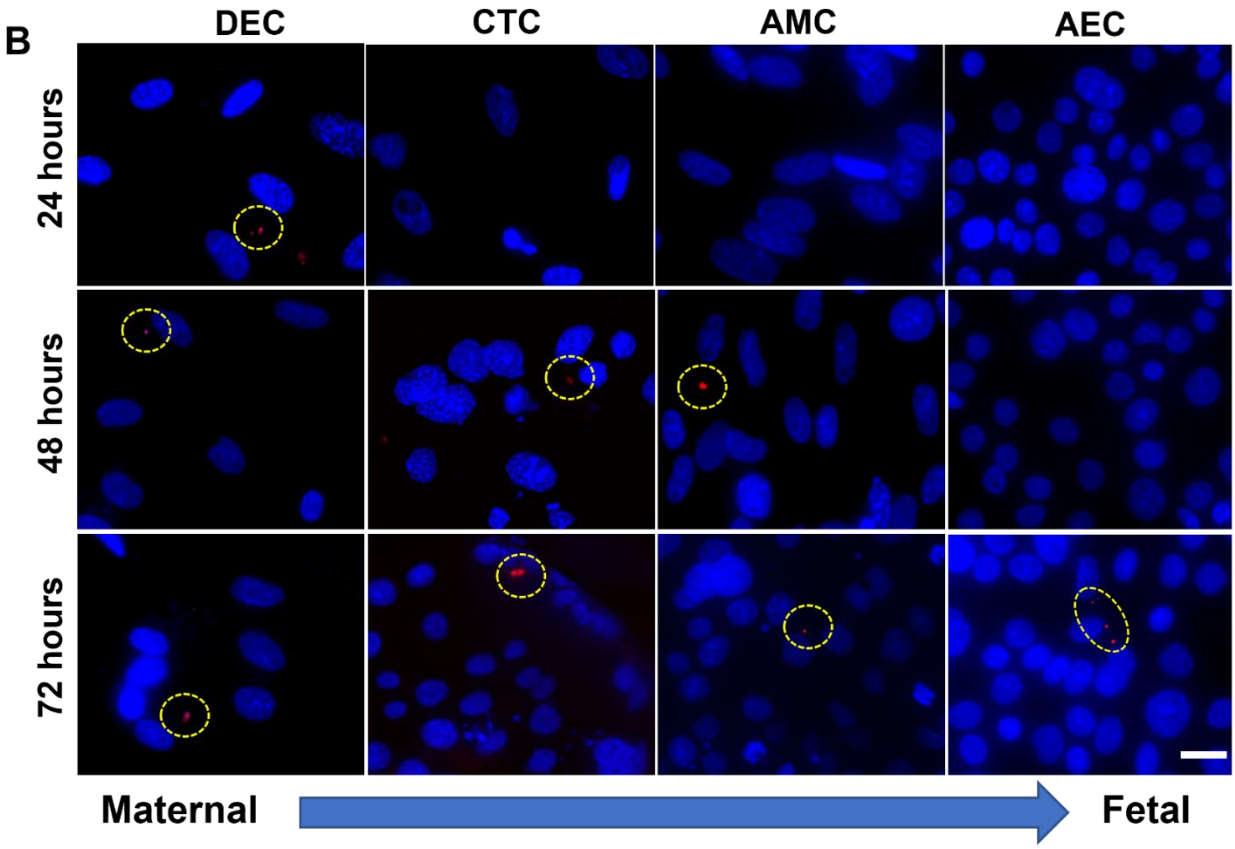


Figure 3. Ascending infection of *U. parvum* in the FMi-OOC.
A) Results of the qPCR for *U. parvum* in the culture media of each chamber after each time point. According to the Cq Mean value of the standard curve and the manufacturer's protocol, DNA copy number was calculated. If Cq >35, the sample was considered negative and the amount of DNA was not calculated. N=3/treatment/time point; Data are presented as mean±SEM**. B)** Immunocytochemical staining of multiple banded antigen (MBA- red) (dotted yellow circle), a virulence factor of *U. parvum.* Nuclei are stained with DAPI. Scale bar 10 um. Nuclei are stained with DAPI, n=3 technical replicates. Cq: quantification cycle. Scale bar, 25 μm.

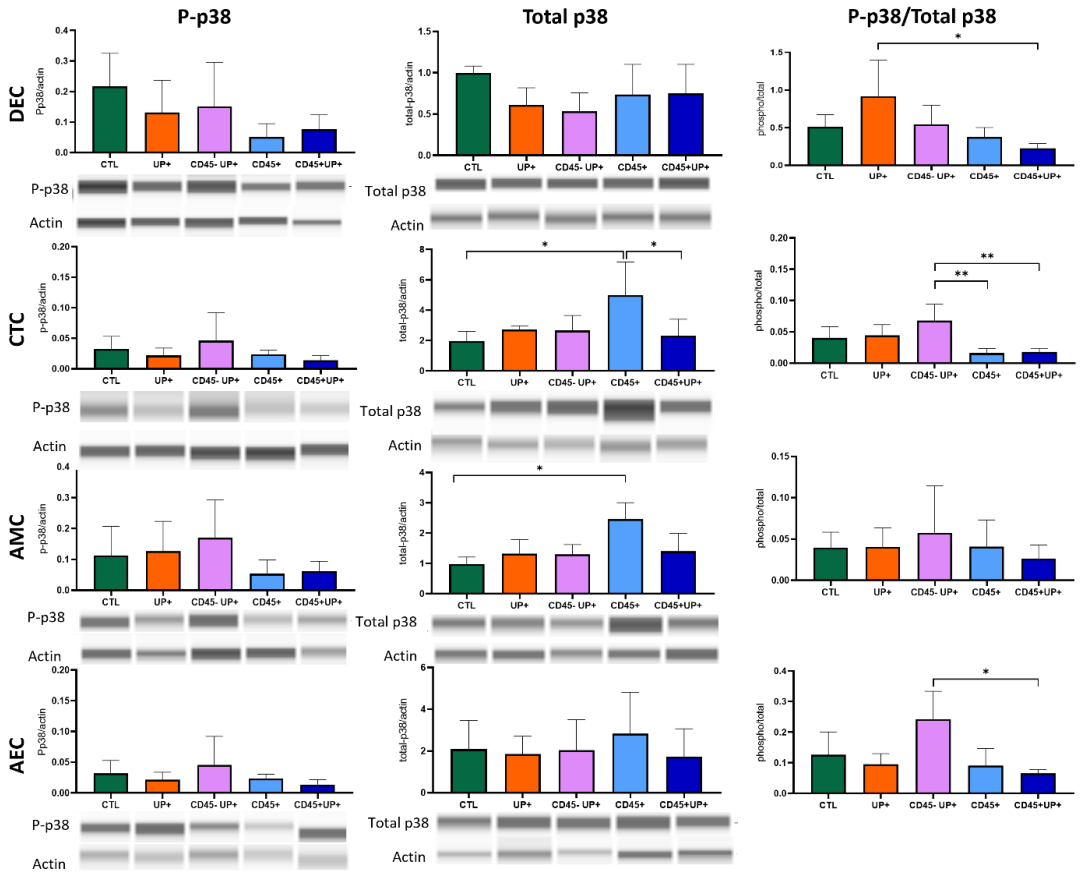


Figure 4. p38 MAPK levels in cell lysates collected from FMiOOC after treatments.

JESS analysis and quantification of phosphorylated p38 MAPK (P-p38 MAPK) and total-p38 MAPK. β-actin was used to normalize the protein concentration. Error bars represent mean ± SEM, n=5. *, $p < 0.05$. **, <0.01. ***,<0.001

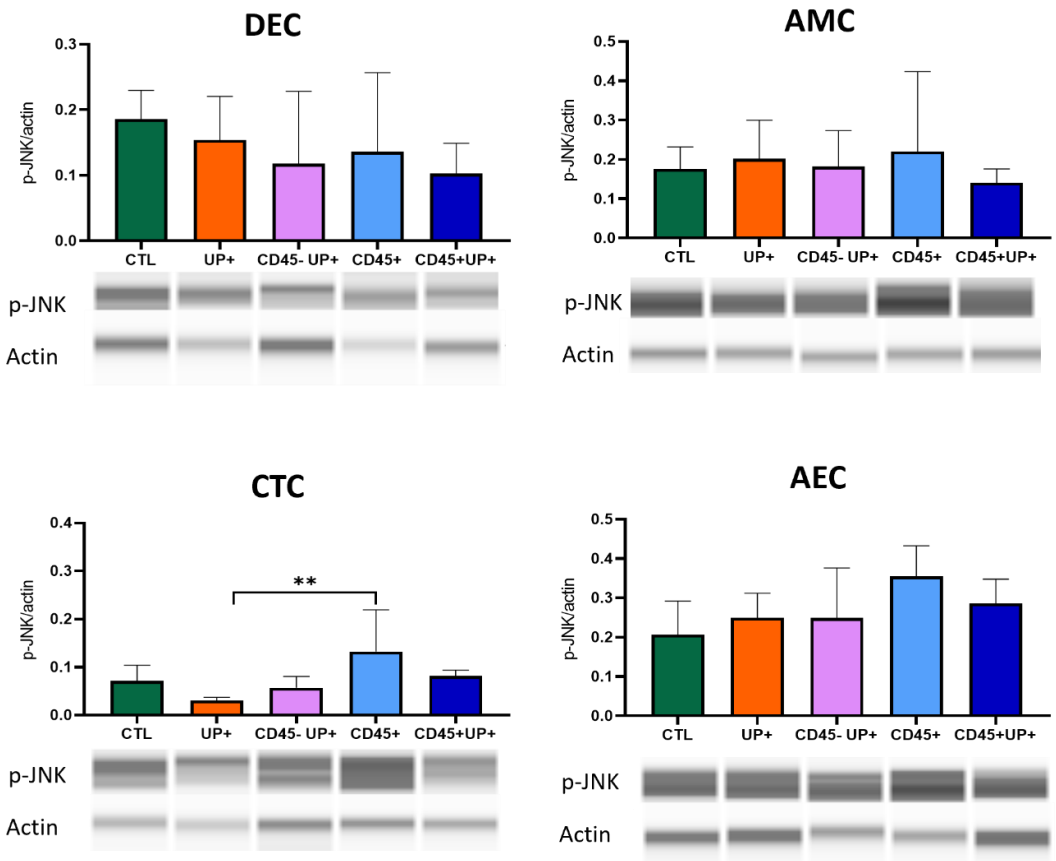


Figure 5. Phospho-JNK levels in cell lysates collected from FMiOOC after treatments.

JESS analysis and quantification of phosphorylated JNK (pJNK). β-actin was used as a control. Error bars represent mean ± SEM, n=5. $p < 0.05$. **,$p < 0.01$. ***,<0.001.

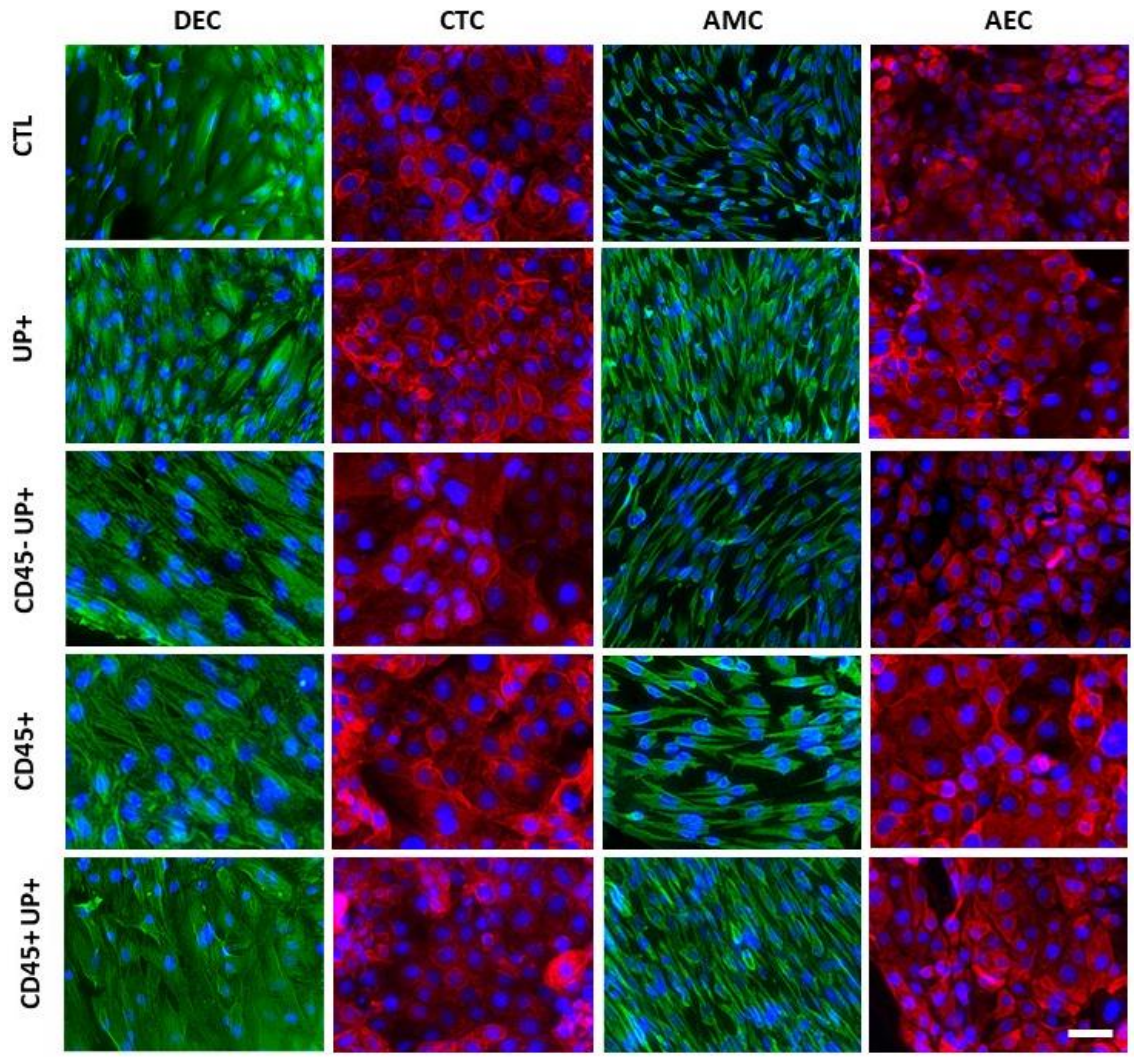


Figure 6. Cell morphology after treatment with *U. parvum* and immune cells. Immunocytochemical staining of the cells in FMi-OOC after 72 hours of treatment. CK-18 (red) was used for amnion epithelial and chorion trophoblast cells while vimentin (green) was used as a marker of amnion mesenchymal and decidual cells. Nuclei were stained with DAPI. N=3 per treatment; 20x magnification. Scale bar: 30 µm. AEC: amnion epithelial cells; AMC: amnion mesenchymal cell; CTC: chorion trophoblast cell; DEC: decidual cells.

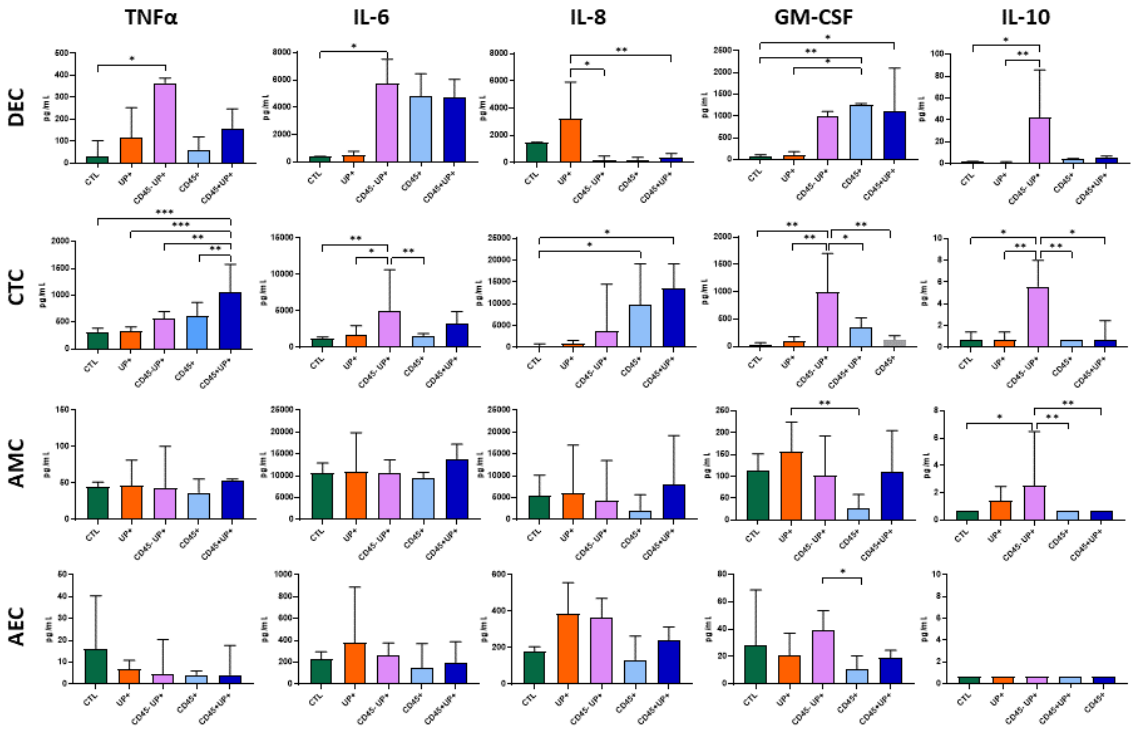
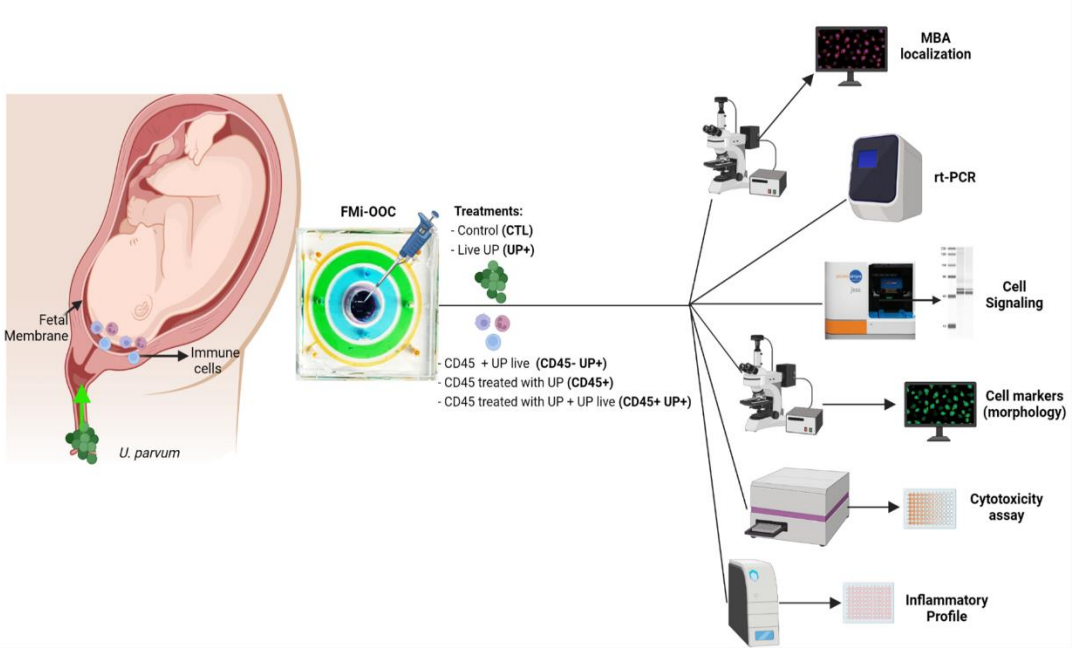


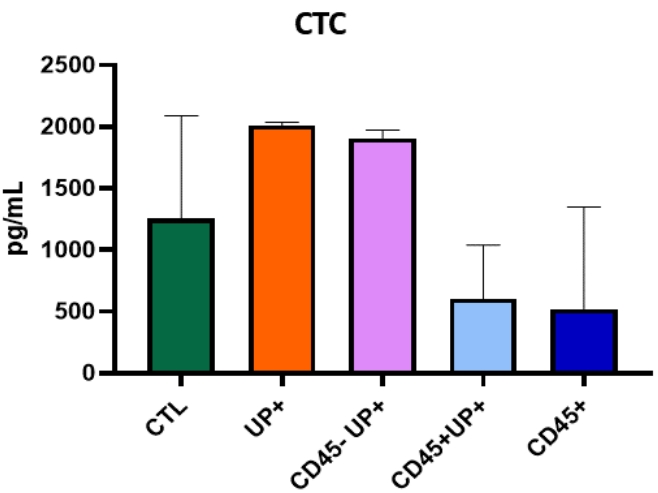
Figure 7. Inflammatory profile of FMiOOC cells after 72 hours in different treatments.

Levels of TNF-α, IL-6, IL-8, GM-CSF, and IL-10 in the FMi-OOC cells measured by Luminex assay. n=5. *, $p < 0.05$. **, <0.01. ***,<0.001.

872 **Supporting Information**
873



874 **Supplementary Figure 1. Study design and methodologies used in the study.**
875 To mimic ascending infection of *U. parvum*, different treatments were added to the DEC
876 chamber of the FMi-OOC: regular DEC media as negative control; live *U. parvum*;
877 supernatant of CD45 cells treated or not with *U. parvum*; live *U. parvum* with CD45
878 supernatant. Different endpoints were included in the study: immunocytochemistry for
879 cell markers and MBA localization; cell signaling analysis; cytotoxicity assay; and
880 inflammatory profile. The schematic design was created using BioRender.
881



882 **Supplementary Figure 2. Progesterone (P4) levels in hFM-CTC cells after**
883 **treatments.**
884 Data are presented as mean ± SEM (N = 5).

Supplementary Table 1. Summary of inflammatory cytokines results.
Summary of the results of each cytokine according to the groups of the study.

Groups	TNFα	IL-6	IL-8	GM-CSF	IL-10
CTL	-	-	-	-	-
UP^{+}	-	-	DEC ↑	AMC ↑	-
CD45^{-}UP^{+}	DEC ↑	DEC ↑ CTC ↑	-	CTC ↑ AEC ↑	DEC ↑ CTC ↑ AMC ↑
CD45^{+}	-	-	CTC ↑	DEC ↑	-
CD45^{+}UP^{+}	CTC ↑	-	CTC ↑	DEC ↑	-
↑ means significantly increased levels of cytokine.					

Capítulo IV

Artigo Científico 2

Manuscrito apresentado de acordo com as normas para submissão no periódico *BMC Pregnancy and Childbirth* (Fator de impacto: 3.8)

Oxidative stress in the amniotic layer associated with polybacterial ascending infection.

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Ethics Statement: *The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.*

Conflict of Interest: The authors declare no conflict of interest in this study.

Abstract

PROBLEM: Inflammation and oxidative stress are associated with spontaneous preterm birth (PTB), which is the leading cause of neonatal mortality and morbidity worldwide. In Latin America, the estimate of PTB was 9.8% of the total pregnancies. Ascending bacterial infection is associated with ~ 40% of spontaneous PTB, being this infection mostly polymicrobial. The fetal membranes play an important role as physical and immunological barrier throughout pregnancy. The objective of this study is to evaluate whether infection of amnion epithelial and mesenchymal cells can cause lipid and protein peroxidation leading to oxidative stress.

METHOD OF STUDY: Immortalized amnion mesenchymal and epithelial cells (hFM-AMC and hFM-AEC) were treated with *Staphylococcus aureus*, *Streptococcus agalactiae*, *Fusobacterium nucleatum*, and *Peptostreptococcus anaerobius* (10E06 CFU/mL). Media and cell lysate were collected after 24 hours. We determined the impact of bacterial infection on protein (3'NT assay) and lipid (TBARS) peroxidation in both types of cells. In addition, total antioxidant capacity (Antioxidant Kit) was measured at this time point.

RESULTS: hFM-AEC showed higher levels of 3-NT in the treatment groups with facultative anaerobic ($p<0.05$), strict anaerobic ($p<0.05$), and all species ($p<0.05$) compared to the control group. hFM-AEC showed significant elevated levels of MDA only in the group with stimulation of all species ($p<0.05$) compared to all the other groups. hFM-AMC levels of both markers did not change between the groups. hFM-AMC showed significantly lower levels of antioxidant capacity in the facultative anaerobic and all species group ($p<0.05$).

CONCLUSIONS: Infection of species that are commonly associated with spontaneous preterm birth can cause oxidative stress in the amnion epithelial cells but did not affect the amnion mesenchymal cells. However, the total antioxidant capacity after infection has changed in the amnion mesenchymal cells. Our data highlight the different contributions that each type of cell that composes the feto-maternal interface has in the mechanisms that trigger preterm birth.

Keywords: preterm birth; oxidative stress; ascending infection; polybacterial infection; lipid peroxidation; protein peroxidation.

Introduction

Annually, 15 million babies are born preterm [1], defined by the delivery that occurs before 37 weeks of gestation. Preterm birth (PTB) is the leading cause of neonatal mortality and morbidity worldwide. In Latin America, the estimate was 9.8% of the total pregnancies [2]. In Brazil, the preterm birth rate was 11.2%, representing 2.3% of the global proportion of cases [3, 4]. The etiology of spontaneous PTB is multifactorial, including genetics, previous history of PTB, and habits. However, 40% of the total of PTB is associated with ascending bacterial infection [5].

The fetal membranes play an important role during pregnancy. Among different functions, they are a physical barrier between mother and fetus, maintain the sterile condition of the amniotic fluid, and sustain an immunological barrier in the feto-maternal interface [4, 6, 7]. The amniotic layer is composed by epithelial and mesenchymal cells and is in contact with the amniotic fluid [8]. The presence of Toll-like receptors (TLR) in this layer suggests the involvement in the inflammatory response during pregnancy [6]. It is crucial to better understand the contribution of the amniotic cells in the processes that lead to preterm birth.

In the majority of cases, ascending infection is polybacterial by species

that are associated with bacterial vaginosis, such as *Gardnerella vaginalis*, *Peptostreptococcus anaerobius*, *Streptococcus agalactiae* and genital mycoplasmas, including *Mycoplasma hominis*, *Ureaplasma urealyticum,* and *U. parvum* [9-12]. Considering the polybacterial nature of ascending infection, it is crucial to study the complexity of microbiological aspects and pathogenic mechanisms, as interactions between species can lead to distinct clinical outcomes [13]. It is well stablished that the ascending infection can trigger the inflammatory response that lead to preterm labor [14-17]. High levels of pro-inflammatory cytokines, such as Interleukin (IL)-6, IL-8, Tumor necrosis factor (TNF) - α were associated with PTB , preceded or not by preterm premature rupture of membranes (PPROM) [18, 19].

In this context, oxidative stress (OS), defined by an imbalance between the accumulation of reactive oxygen species (ROS) is considered a physiological process in the context of pregnancy when it occurs after 37 weeks of gestation [20]. In addition to the increase of ROS, the depletion of antioxidant system favors the scenario of oxidative stress [21]. This process can also lead to telomere shortening through the response to DNA damage [22], resulting in early telomere shortening can lead to PTB [23]. There is a strong correlation between oxidative stress and inflammation, since H_2O_2 can activate nuclear factor kappa B (NFκB) pathway, resulting in proinflammatory genes activation [21, 24]. Antioxidant mechanisms include enzymatic antioxidants, such as catalase, and non-enzymatic antioxidants, such as glutathione [25]. The inflammatory response triggered by bacterial infection is associated with an increased production of ROS, resulting in

oxidative stress [26]. Furthermore, immune cells, such as neutrophils, produce ROS as a consequence of defense mechanisms against infection. In the context of PTB, studies have been reported an association between oxidative stress, senescence, and stress-induced inflammation[24, 27].

Protein and lipid peroxidation are one of signs of oxidative stress. 3-Nitrotyrosine (3-NT) is a product of protein nitration and free tyrosine residues by peroxynitrite molecules, resulting in cytoskeleton damage and changes in protein function [28, 29]. Malondialdehyde (MDA) is product of lipid peroxidation, a mechanism of cellular injury that indicates oxidative stress in cell and tissues. On the other hand, human antioxidant systems include enzymes, such as catalase and glutathione peroxidase, to counteract ROS production. Some studies have described the correlation of the OS and preterm birth [25, 27, 30] but the mechanisms of the process in the polybacterial scenario still unclear.

We hypothesize that facultative aerobic and strict anaerobic bacteria involved in the ascending infection of the female genital tract can cause oxidative stress and alter the antioxidant balance in the amniotic layer. The objective of this study is to evaluate whether infection of amnion epithelial and mesenchymal cells can cause lipid and protein peroxidation leading to oxidative stress.

Methods

1. Cell culture

1.1. Amnion mesenchymal and epithelial cells

Immortalized Human Fetal Membrane – Amnion mesenchymal cells (hFM-AMC) and hFM- Amnion epithelial cells (hFM-AEC) used in this study were a donation from Dr. Ramkumar Menon from University of Texas Medical Branch (Galveston, TX). Fetal membrane cells were isolated from elective term Cesarean section, not in labor placenta, approved by the Institutional Review Board at UTMB, and immortalized as reported previously [31]. hFM-AMC were cultured in Dulbecco's Modified Eagle Media (DMEM)/F12 (Mediatech Inc., USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Mediatech Inc., USA), and 1% amphotericin B (Sigma-Aldrich Corporation, USA), and hFM-AEC were cultured in Keratinocyte serum-free medium (KSFM) supplemented with bovine pituitary extract (30 μg/mL), epidermal growth factor (0.1 ng/mL), $CaCl_2$ (0.4 mM), and primocin (0.5 mg/mL). Both cell types were grown at 37◦C and 5% CO_2.

2. Bacterial strain and culture conditions

Fusobacterium nucleatum (ATCC #25586), *Peptostreptococcus anaerobius* (ATCC #27337), *Staphylococcus aureus* (ATCC #6538), *Streptococcus agalactiae* (ATCC #12386) were obtained from the American Tissue Culture Collection (ATCC). *F. nucleatum* and *P. anaerobius* were cultured in Thioglycollate medium (K25-610050, KASVI) in anaerobic conditions according to manufacturer instructions. *S. aureus* were cultured in Agar Tryptic Soy (K25-1068.11, KASVI), and *S. agalactiae* in Brain Heart Infusion Broth supplemented with Defibrinated Sheep Blood (5%). Bacteria were incubated for 48h. The

colony-forming units (CFU) were determined by McFarland scale 4 (1x10E09 CFU/mL).

3. Bacterial stimulation of AEC and AMC

hFM-AECs and hFM-AMC were seeded at 800,000 cells/ T25 flasks. After 24h, cell cultures were stimulated with 10^6 CFU of bacterial suspension diluted in supplemented KSFM and supplemented DMEM/F-12, respectively. Treatments were as followed (Table 1): 1) regular hFM-AEC and hFM-AMC media as a negative control (CTL-) (n=5); 2) 100ng/mL of LPS from *E. coli* (O55:B5 - Sigma-Aldrich Corporation, USA) as a positive control (CTL+) (n=5); 3) live *S. aureus* and *S. agalactiae* at 10E06 CFU/mL concentration to mimic the scenario of ascending infection by facultative anaerobic bacteria (Fac. Anaer.) (n=5); 4) live *P. anaerobius* and *F. nucleatum* at 10E06 CFU/mL concentration to mimic the scenario of ascending infection by strict anaerobic bacteria (Anaer.) (n=5); 5) live *S. aureus, S. agalactiae, P. anaerobius* and *F. nucleatum* at 10E06 CFU/mL concentration to mimic the scenario of polybacterial infection (All) (n=5). Treated cells were incubated at 37°C, 5% CO_2 for 24h. After 24h, media from all T25 flasks were collected and stored at -80°C, and cells were harvested with a rubber policeman and collected with 1mL of either cold PBS or appropriate media.

Table 1: Different types of treatments in the *in vitro* stimulation of hFM-AEC and hFM-AMC.

Groups	Treatment	Specification
Group 1	CTL -	Regular hFM-AEX or hFM-AMC media (control)
Group 2	CTL +	LPS of *E. coli* at a 100ng/mL concentration
Group 3	Facultative Anaerobic (Fac. Anaer.)	Live *S. aureus* and *S. agalactiae* at a concentration of 10E06 CFU/mL
Group 4	Anaerobic (Anaer.)	Live *P. anaerobius* and *F. nucleatum* at a concentration of 10E06 CFU/mL
Group 5	All	Live *S. aureus, S. agalactiae, P. anaerobius,* and *F. nucleatum* at a concentration of 10E06 CFU/mL

4. Measurement of 3-nitrotyrosine levels

Protein peroxidation was determined using the 3-NTnitrotyrosine (3-NT) ELISA Kit, according to the manufacturer's instructions (Cayman Chemical, Michigan, US). The supernatant of each sample was collected after 24 hours from different treatments and centrifuged for 20min at 1000g at 2-8^{o}C. A standard curve was obtained in parallel with the samples to each assay and the absorbance results were converted to ng/mL. The absorbance of samples and standard curve were read at 450nm in an automatic ELISA reader (Biotek Instruments Inc, USA). The minimum detectable 3-NT level was 1.56 ng/mL. The intra and inter-assay variability rates were < 10.0%.

5. Measurement of Malondialdehyde

Levels of lipid peroxidation were determined by measurement of

Thiobarbituric acid reactive substances (TBARS) using TBARS (TCA Method) Assay Kit (Cayman Chemical, Michigan, US). The assay was followed according to manufacturer's instructions. Briefly, cell lysate for each sample and treatment was mixed in a 2.0mL screw cap vial with TCA Assay Reagent (10%) and Color Reagent. Vials were boiled for one hour, transferred to an ice bath for 10 minutes, centrifuged for 10 minutes at 1,600g at 4^{o}C, and 200ul of the supernatants were added do a 96well-plate. A standard curve was obtained to each assay and the absorbance results were converted to μM/mL. The absorbance of samples and standard curve was read at 530nm in an automatic ELISA reader (Biotek Instruments Inc, USA). The minimum detectable MDA level was 0.084 μM/mL. The intra and inter-assay variability rates were < 10.0%.

6. Measurement of total antioxidant capacity

The total antioxidant capacity was measured using Antioxidant Kit, according to manufacturer's instructions (Cayman Chemical, MI, USA), assessing combined compounds of the antioxidant system. The methodology of the assay consists on the ability of the antioxidants presented in each sample to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) to ABTS+ by metmyoglobin. Briefly, cell lysates from both type of cells were collected after 24 hours, sonicated, and centrifuged. Supernatant of the cell lysate was mixed with metmyoglobin, chromogen and hydrogen peroxide. A Trolox standard curve was obtained, and results were expressed as millimolar Trolox equivalent (mM Trolox). The absorbance of samples and standard curve was read at 750nm in an

automatic ELISA reader (Biotek Instruments Inc, USA).

7. Statistical analysis

The Shapiro-Wilk test was used to check normality. Parametric data were analyzed by one-way ANOVA followed by Tukey comparison post hoc. For non-parametric data, Kruskal-Wallis test was used followed by Dunn's multiple comparisons. A p value <0.05 was considered statistically significant. The software used was GraphPad Prism software version 8 (GraphPad Software, CA, USA).

Results

1. Quantification of oxidative stress by protein peroxidation

As bacterial infection is linked to inflammatory response [32], we investigated if polybacterial infection can be associated with different oxidative stress levels. Protein peroxidation was measured by 3-NT concentrations. There were no significant changes in the level of 3-NT in hFM-AMC between the groups. However, hFM-AEC showed higher levels of 3-NT in the treatment groups with Facultative Anaerobic ($p<0.05$), Anaerobic ($p<0.05$), and All species ($p<0.05$) compared to the control groups (Figure 1). There were no significant changes among the different treatment groups, suggesting that between the two types of cells that form the amnion layer, only epithelial cells are associated with increased protein peroxidation induced by polybacterial infection, independent of species requirements.

2. Quantification of oxidative stress by lipid peroxidation

Lipid peroxidation was measured by levels of MDA, one of the final products of polyunsaturated fatty acids peroxidation in cells, and the most common used biomarker[33]. hFM-AMC levels of MDA were overall high but did not show significant changes of this marker among the groups (Figure 2). On the other hand, hFM-AEC showed significant elevated levels of MDA only in the group with stimulation of all species ($p<0.05$) compared to all the other groups, although levels were low. The other groups, including CTL+ (positive control) did not show high levels of MDA concentration. These findings suggest the potential of amnion epithelial cells to induce lipid peroxidation by bacterial infection.

3. Antioxidant capacity

To evaluate whether the combination of the bacterial species used in this study can affect the antioxidant capacity of amnion cells, the total antioxidant capacity was measured by ELISA assay. hFM-AMC showed significantly lower levels of antioxidant capacity in the facultative anaerobic and all species group compared to the positive control ($p<0.05$) (Figure 3). However, there were no significant changes between the different treatment in hFM-AEC. The data suggest that although protein and lipid peroxidation were seen in hFM-AEC, the antioxidant capacity had only been affected in the hFM-AMC.

Discussion

The main pathways that lead to labor and delivery include oxidative stress and

inflammation. ROS are physiologically produced as part of aerobic energy process, balanced by antioxidant systems [30]. Nevertheless, the redox imbalance can lead to cellular damage due to the presence of ROS that were not eliminated by the system. In this study we demonstrated that bacterial species that are commonly associated with PTB can cause oxidative stress in the amniotic layer. The main findings are: (1) Lipid and protein peroxidation caused by bacterial infection had significant changed levels in amnion epithelial cells; (2) Polybacterial infection with *S. aureus, S. agalactiae, F. nucleatum, P. anaerobius* induced lipid peroxidation in hFM-AEC after 24 hours; and (3) Both facultative anerobic and anaerobic bacteria resulted in lower levels of total antioxidant capacity in amnion mesenchymal cells. These findings suggest that the ascending bacterial infection, considered one of the major risk factors for PTB is associated with oxidative stress markers and antioxidant capacity.

Intra-amniotic infection and inflammation corresponds to ~50% of the total of PTB. The high rates of this association demonstrate the importance of studies that aim to evaluate the role of different species in this scenario. Although in this study we did not see differences between facultative anaerobic and anaerobic species, we have evidence in the literature that *S. agalactiae, S. aureus* were the most prevalent species in cases of preterm birth [34].

Protein peroxidation compromise cell function and affects permeability and integrity [35]. 3-NT is a marker of protein modification caused by OS. Menon et al. identified higher levels of this marker in fetal membranes of from preterm

premature rupture of membranes (pPROM) compared to PTB [36]. Study using fetal membrane tissue culture did not have differences in 3-NT levels between term and preterm birth group [37]. Our findings show that each type of cell that compose the amnion layer is affected differently depending on the infection. Although the levels of total antioxidant capacity remained unchanged after infection in hFM-AEC, these species had the potential to induce protein peroxidation.

It is well stablished that high levels of ROS affect the lipid metabolization and cause lipid damage, leading to lipid peroxidation [33]. High levels of MDA, a product of lipid peroxidation, was observed in PTB group when compared to term birth [38]. In addition, MDA concentration of plasma samples from very low birth weight preterm infants were higher when compared to cord blood from term infants [39]. This was the first time that we studied each type of cell of the amniotic layer separately, showing differences in the way that they are affected by infection, and we suggest that the bacterial load is associated with increased levels of lipid peroxidation in hFM-AEC.

Both protein and lipid peroxidation were altered upon infection in the hFM-AEC, the outermost type of cell of the fetal membranes, which is in contact with the amniotic fluid. The amniotic layer includes two types of cells: the epithelial and mesenchymal cells. Both of them are responsible for production of different growth factors, cytokines, and extracellular matrix [40]. The amnion epithelial cells have the cuboidal morphology and the epithelial to mesenchymal transition

(EMT) happen to these cells upon inflammation [41]. Study have shown that hFM-AMC are able to secrete some anti-inflammatory cytokines such as prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), and transforming growth factor beta (TGF-β) [42]. This anti-inflammatory property might be the reason for the mesenchymal cells not having lipid and protein peroxidation altered levels after bacterial infection. Although there are studies that takes into consideration the whole fetal membranes, our findings highlight the importance of considering the role of each cell that compose the feto-maternal interface, especially the cells of the amniotic layer.

Antioxidants are defined as substances that protect the cells from the damage that can be caused by free radicals and they can be divided into enzymatic, the first line of defense, or non-enzymatic [43]. Since oxidative stress is a consequence of the redox imbalance, the reduction of the total antioxidant capacity has its importance in this scenario. In this study we found that the levels of total antioxidant capacity in amnion epithelial cells have not changed in the presence of either facultative anaerobic or anaerobic bacteria. However, the antioxidant capacity after infection was reduced in the amnion mesenchymal cells, showing that the mechanisms associated to the antioxidant capacity in this type of cells may not be as effective as in epithelial cells. In normal pregnancies, total antioxidant capacity levels in amniotic fluid were detected early in the second trimester and increased throughout the pregnancy [44].

At term, oxidative stress is a well-understood condition, caused by

physiological aging of the placenta [36]. Here, we demonstrated the protein and lipid peroxidation in the amniotic layer caused by bacterial infection that are associated with PTB, suggesting a mechanism of pathogenesis that can lead to this adverse outcome. Previous study showed that different sites of the fetal membranes can have distinct antioxidant enzyme activity being more susceptible to oxidative damage [25]. This study has some limitations, including 1) not using genital mycoplasmas species to evaluate their impact in oxidative stress, though the species chosen was based on the most common species found in the amniotic fluid [10], 2) not checking different time points and long term infection as seen *in vivo*, and 3) lack of NFκB analysis since it is a pathway associated with oxidative stress. To address the short comings of this study, further studies will be conducted. Polybacterial infection can cause oxidative stress in the amniotic layer and each cell type of this layer is affected in a different manner.

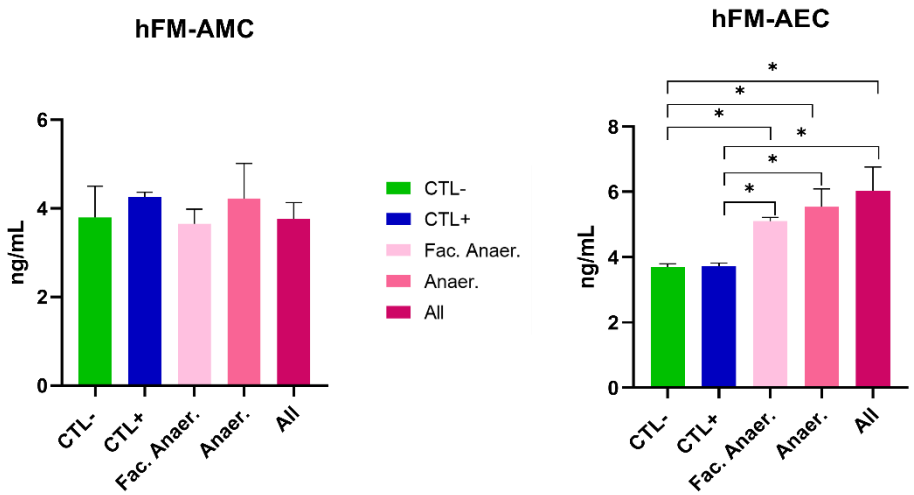
Conclusion

We concluded that infection of species that are commonly associated with preterm birth can cause oxidative stress in the amnion epithelial cells by lipid and protein peroxidation but did not affect the amnion mesenchymal cells. However, the total antioxidant capacity after infection has changed in the amnion mesenchymal cells. We suggest that bacterial load is determinant to trigger lipid peroxidation in amnion mesenchymal cells, and the antioxidant capacity is not as effective as seen in amnion epithelial cells. Our data support the importance of the

studies with different cell types that are part of the feto-maternal interface and highlight the crucial role of these cells in the context of preterm birth.

Figures

Figure 1. Protein peroxidation measured by 3-nitrotyrosine levels in hFM-AEC



and hFM-AMC. 3-NT assay was performed with the media of both types of cells in every treatment after 24 hours. Data are presented as mean±SEM. N=5. *, $p < 0.05$. CTL -: negative control; CTL+: positive control; Facultative anaerobic: *Staphylococcus aureus* + *Streptococcus agalactiae*; Anaerobic: *Peptostreptococcus anaerobius* and *Fusobacterium nucleatum;* All*: S.aureus, S. agalactiae, P. anaerobius,* and *F. nucleatum*

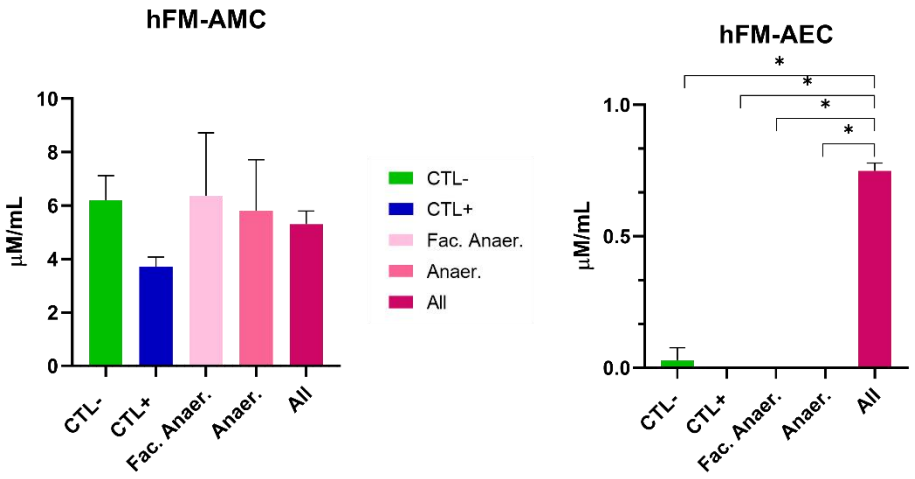


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Figure 2. Lipid peroxidation measured by MDA levels in hFM-AEC and hFM-AMC. Lipid peroxidation was measured by MDA levels by ELISA with cell lysates of both types of cells in every treatment after 24 hours. Data are presented as mean±SEM. N=5. *, $p < 0.05$. CTL -: negative control; CTL+: positive control; Facultative anaerobic: *Staphylococcus aureus* + *Streptococcus agalactiae*; Anaerobic: *Peptostreptococcus anaerobius* and *Fusobacterium nucleatum;* All*: S.aureus, S. agalactiae, P. anaerobius,* and *F. nucleatum.*

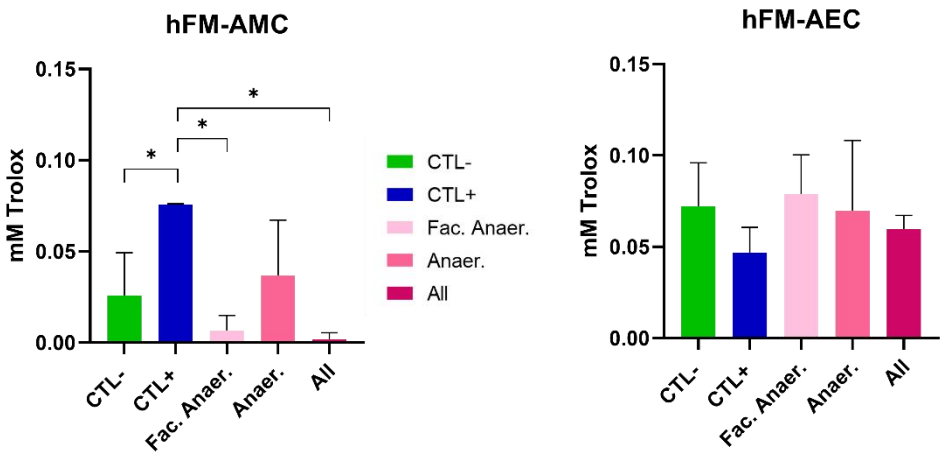

Figure 3. Total antioxidant capacity in hFM-AEC and hFM-AMC. Antioxidant capacity was assessed by Antioxidant ELISA Kit with the cell lysate of both types of cells in every treatment after 24 hours. Data are presented as mean±SEM. N=5. *, $p < 0.05$. CTL -: negative control; CTL+: positive control; Facultative anaerobic: *Staphylococcus aureus* + *Streptococcus agalactiae*; Anaerobic: *Peptostreptococcus anaerobius* and *Fusobacterium nucleatum;* All*: S.aureus, S. agalactiae, P. anaerobius,* and *F. nucleatum.*

List of abbreviations:

3-NT: 3-Nitrotyrosine

ATCC: American Tissue Culture Collection

CFU: Colony-forming units

DMEM: Dulbecco's Modified Eagle Media

EMT: Epithelial to mesenchymal transition

hFM-AEC: Amnion epithelial cells

hFM-AMC: Amnion mesenchymal cells

HGF: Hepatocyte growth factor

IL: Interleukin

KSFM: Keratinocyte serum-free medium

MDA: Malondialdehyde

NFκB: nuclear factor kappa B

PGE2: Prostaglandin E2

PPOM: Preterm premature rupture of membranes

PTB: Preterm birth

OS: Oxidative stress

ROS: Reactive oxygen species

TBARS: Thiobarbituric acid reactive substances

TGF-β: Transforming growth factor beta

TLR: Toll-like receptors

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Conclusão final

O parto pré-termo espontâneo está relacionado com a infecção bacteriana ascendente do trato genital inferior que culmina na infecção da interface materno-fetal. Essa infecção, na maioria das vezes de caráter polibacteriano e associada a micoplasmas genitais, tem estreita relação com a resposta inflamatória. Nesse trabalho foi possível validar o modelo de *organ-on-chip* que mimetiza a interface materno-fetal para estudos da infecção ascendente do trato genital feminino. Esse avanço possibilita que novos estudos aconteçam acerca dessa interface que tem papel crucial ao longo da gestação. Resultados usando esse modelo mostram que a espécie *U. parvum* não é imunogênica o suficiente para deflagrar resposta inflamatória e causar alterações em vias de sinalização quando sozinho na interface materno-fetal.

O estudo com infecção polibacteriana de espécies anaeróbias facultativas e estritas na camada amnióticas das membranas fetais mostrou que a infecção esteve associada à peroxidação lipídica e proteica em células amnióticas epiteliais, enquanto que a capacidade antioxidante total foi diminuída nas células amnióticas mesenquimais frente à infecção. Esses achados ressaltam a importância que cada célula que compõe as membranas fetais tem no contexto da gestação, e que estudos acerca de cada tipo celular são fundamentais para o melhor entendimento dos processos nessa interface.

Em conjunto, os achados apresentados nesse trabalho reforçam a hipótese de que a infecção bacteriana ascendente tem participação na

resposta inflamatória, senescência e estresse oxidativo. Juntos, esses processos estão associados a deflagração do parto pré-termo. Porém, essa associação vai depender da espécie em questão, da carga bacteriana e do tipo de combinação polibacteriana, visto que cada espécie pode deflagrar respostas distintas. O estudo do efeito e da associação de cada espécie bacteriana nesses processos corrobora para que estratégias sejam desenvolvidas na clínica obstétrica para que esse desfecho gestacional adverso tenha sua prevalência reduzida.