UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

ESTUDO COMPARATIVO DO PERFIL IMUNOFENOTÍPICO, POTENCIAL DE DIFERENCIAÇÃO, CAPACIDADE DE PRODUÇÃO DE CITOCINAS E CRIOPRESERVAÇÃO DE CÉLULAS ESTROMAIS DO ENDOMÉTRIO DE VACAS DURANTE O CICLO ESTRAL

CAROLINA NOGUEIRA DE MORAES MAIA

Botucatu, São Paulo
Agosto, 2017

UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

ESTUDO COMPARATIVO DO PERFIL IMUNOFENOTÍPICO, POTENCIAL DE DIFERENCIAÇÃO, CAPACIDADE DE PRODUÇÃO DE CITOCINAS E CRIOPRESERVAÇÃO DE CÉLULAS ESTROMAIS DO ENDOMÉTRIO DE VACAS DURANTE O CICLO ESTRAL

CAROLINA NOGUEIRA DE MORAES MAIA

Tese apresentada à Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus Botucatu, para a obtenção do título de Doutor em Biotecnologia Animal, área de Reprodução Animal.

Orientadora: Profª Dr. Eunice Oba

Botucatu, São Paulo
Agosto, 2017

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Maia, Carolina Nogueira de Moraes.
Estudo comparativo do perfil imunofenotípico, potencial de diferenciação, capacidade de produção de citocinas e criopreservação de células estromais do endométrio de vacas durante o ciclo estral / Carolina Nogueira de Moraes Maia. - Botucatu, 2017

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina Veterinária e Zootecnia
Orientador: Eunice Oba
Capes: 50504002

1. Bovino. 2. Ciclo estral. 3. Células-tronco. 4. Células mesenquimais estromais - Criopreservação. 5. Citocinas. 6. Endométrio. 7.Útero.

Palavras-chave: LPS; Bovinos; CTMsE; Caracterização; Útero.

Nome da Autora: Carolina Nogueira de Moraes Maia
Data da Defesa: 31 de agosto de 2017.

Banca Examinadora:

____

Profa. Tit. Eunice Oba
Presidente e Orientadora
Depto. de Reprodução Animal e Radiologia Veterinária
FMVZ – UNESP- Botucatu

____

Prof. Dr.Rogério Martins Amorim
Membro
Depto de Clínica Veterinária
FMVZ – UNESP- Botucatu

____

Prof.Dra. Fernanda da Cruz Landim
Membro
Depto. de Reprodução Animal e Radiologia Veterinária
FMVZ – UNESP- Botucatu

____

Prof.Dr. Carlos Eduardo Ambrósio
Membro
Depto. de Medicina Veterinária
FZEA - Universidade de São Paulo – São Paulo

____

Prof.Dr. Armando de Mattos Carvalho
Membro
Depto. Clínica e Cirurgia Veterinárias
Escola de Veterinária, UFMG – Belo Horizonte

DEDICATÓRIA

Ao Autor da Vida, sempre!

Agradecimentos

Inicialmente à Deus, por me fortalecer e me mostrar que Ele sempre esteve, está e estará ao lado, me iluminando e sustentando. A jornada foi longa e árdua, mas nunca me senti só.

Agradeço meu esposo Leandro, pelo incentivo e por estar ao meu lado em todas as etapas da minha vida. Todas as dificuldades e vitórias que alcançamos juntos são especiais e foram guiadas por Deus. Te amo.

Agradeço aos meus pais Salviana e Enio por acreditarem sempre em mim e me fazerem cada dia mais forte, mesmo sem saberem. Mãe, que este seja mais um presente de Deus na sua nova vida.

Agradeço às minhas irmãs Daniela e Camila e aos meus cunhados Jr. e Joelson pelo suporte e pela retaguarda.

Agradeço meus sobrinhos Manuela e Arthur por serem meus amores e me encherem de alegria todos os momentos que estamos juntos. E por me matarem de saudade todos os momentos que estamos separados.

Agradeço aos irmãos que Deus permitiu que eu escolhesse: Caroline Destro, Carla Queiroz, Pedro Orlandini, Marianne Camargos, Paula Grippa pela amizade e cumplicidade.

Agradeço aos amigos que pude fazer em Barcelona durante o doutorado sanduíche: Lia, Julia, Cris e Jerônimo. Obrigada por fazerem deste tempo nesse lugar maravilhoso, ainda mais especial e produtivo.

Agradeço a todos os colegas do laboratório, em especial à Marianne Camargos e Caroline Geraldini por estarem na retaguarda quando precisei. Agradeço aos funcionários do departamento de Reprodução Animal pela amizade e conversas.

Agradeço aos professores do departamento que desde muito tempo me acompanham, ensinam e inspiram.

Agradeço à Prof. Fernanda Landim pelo suporte e por abrir tão prontamente as portas do laboratório para desenvolvermos esse experimento.

Agradeço minha orientadora Dra. Eunice Oba por acreditar no meu trabalho e apoiar-me desde o Mestrado. Tudo foi mais leve com sua compreensão e incentivo.

Agradeço ao convênio FAPESP/CAPESP pela bolsa de estudos (2014/20447-2, 2015/18964-1 Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) e auxílio financeiro (2015/01057-1 Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

Por fim agradeço aos animais, peças chaves em minha vida.

“Não fui eu que lhe ordenei? Seja forte e corajoso! Não se apavore, nem se desanime, pois, o Senhor, o seu Deus, estará com você por onde você andar."
Josué 1:9

LISTA DE ABREVIAÇÕES

CD: cluster of differentiation

CT: células - tronco

CTM: células tronco de origem mesenquimal

CTMsE: células - tronco de origem mesenquimal do tecido endometrial

DMSO: dimetilsulfóxido

IDO: indoleamina 2,3 dioxygenase

INF-γ : interferon γ

LPS: lipopolissacarídeo

MC: meio condicionado

MIP-1α: proteína inflamatória de macrófago 1α

MIP-1β: proteína inflamatória de macrófago 1β

MS: espectometria de massas

NO: óxido nítrico

PCR: reação em cadeia da polimerase

PGE_2: prostraglandina E_2

SFB: soro fetal bovino

TLR: toll-like receptor

TNF-α: fator de necrose tumoral α;

TNF-β: fator de necrose tumoral β

UFC-F: unidades formadoras de colônias fibroblastóides

LISTA DE TABELAS

CAPÍTULO 1

Tabela 1 – Data before and after cryopreservation analysis on flow cytometry using anexin V an propidium iodide (PI) from samples of bovine endometrial mesenchymal stem/progenitor cells from Phase II and Phase III. Data is presented as mean and SEM... 58

CAPÍTULO 2

Tabela 1 – Protein identified in the secretome of conditioned medium of bovine eMSCs treated or not with LPS……............................ 77

CAPÍTULO 3

Tabela 1 – Sequence of primers, annelling temperature and amplification product sizes of genes used to evaluate bovine eMSCs.…….. 88

LISTA DE QUADROS

CAPÍTULO 1

Quadro 1- Painel de marcadores de superfície e intracelular já descritos em cultivos de CTMsE em diferentes espécies por citometria de fluxo ou PCR............................……………………............. 12

LISTA DE FIGURAS

Figura 1 - Esquema da classificação das CT com relação à origem e potencial de diferenciação. Fonte: arquivo pessoal 7

Figura 2 – Modelo de ativação das MSCs por citocinas pro inflamatórias ou por TLRs. Ativação dos perfis anti-inflamatório (CTM 2) ou pró-inflamatórios (CTM 1). Adaptado de Bernardo & Fibbe, 2013. INF-γ: interferon γ; TNF-α: fator de necrose tumoral α; TNF-β: fator de necrose tumoral β; CTM: célula tronco mesenquimal; TLR: toll-like receptor; NO: óxido nítrico; IDO: idolamina 2,3 dioxygenase; LPS: lipopolissacarídeo bacteriano; CYCL9; CXCL10; MIP-1α: proteína inflamatória de macrófago 1α; MIP-1β: proteína inflamatória de macrófago 1β. 20

CAPÍTULO 1

Figura 1 - Immunophenotypic analysis for the markers CD44, CD29, vimentin, CD34, and MHC-II from samples of bovine eMSCs from Phase II (A, C, E, G, I) and Phase III (B, D, F, H, J). Representative histograms of marker CD44 from phase II (A) and III (B). Representative histograms of marker CD29 from phase II (C) and III (D). Representative histograms of marker vimentin from phase II (E) and III (F). Representative histograms of marker CD34 from phase II (G) and III (H). Representative histograms of marker MCH-II from phase II (I) and III (J). Representative histograms of isotype control IgG from phase II (K) and III (M). Representative histograms of secondary control from phase II (L) and III (N). Data is presented as mean and SEM. There were no differences between groups (p>0.05)………………………………………….. 55

Figura 2 - Immunohistochemistry of bovine eMSCs for characterization of the markers CD44 (A), vimentin (B) and cytokeratin (C). The positive staining is shown in brown. Negative control (D) by omission of the primary antibody. Nucleus stained with hematoxylin. Bar = 50 μm (A.B.C), 100 μm (D)…………………. 56

Figura 3 - Differentiation assay for adipogenic and osteogenic lineages of bovine eMSCs. Adipogenic differentiation: Note in (A) the presence of intracytoplasmic lipids droplets stained with Oil red (arrow) and (B) control of differentiation. Osteogenic differentiation: Note in large calcium deposit stained with Alizarim Red (2%) (arrow) (C) and (D) control of differentiation. Bar: 100 μm (A, C) 200 μm (B, D)……………………………… 57

Figura 4 – (A) Clonicity efficiency of bovine eMSCs. Note a formation of a colony well defined. (B) Karyotype analysis of bovine eMSCs (2n=60)…………………………………………………………… 57

CAPÍTULO 2

Figura 1 - Characterization of bovine eMSCs. Bovine eMSCs on first passage presenting fiblobastoid morfology and adherence to plastic (A). Differentiation assay into osteogenic lineage of bovine eMSCs in third passage (B). Note calcium deposit stained with Alizarim (arrow). Differentiation assay into adipogenic lineage of bovine eMSCs in third passage (C). Note the intracytoplasmic lipids droplets stained with Oil red (arrow). Immunohistochemistry of bovine eMSCs for CD44 (D), vimentin (E) e pancytokeratin (F). Positive staining is observed in brown. Nucleus stained with hematoxylin. Size: 100 μm (A, D, F), 200 μm (B, C, E)……………………………… 74

Figura 2 - Representative histogram for the markers analyzed by flow cytometry on bovine eMSCs: vimentin (A), CD29 (B), CD44 (C), CD34 (D), OCT-4 (E) and MHC-II (F)……………………… 75

Figura 3 – Venn Diagram of proteins identified with at least one peptide sequence (False discovery rate≤1%) on studied groups. TG: treated group; CG: control group………….............................. 76

CAPÍTULO 3

Figura 1 – Timeline. Endometrial mesenchymal stem cells were collected and characterized. Cells on third passage were submitted to quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) and to challenge (TG) or not (CG) with bacterial lipopolysaccharide (LPS). Conditioned medium (CM) was collected after 2, 6, 12 and 24 for cytokine and PGE2 analysis…………………………………………………………....... 85

Figura 2- Ratio of genes/GAPDH mRNA abundance measured by quantitative reverse transcriptase–polymerase chain reaction analysis in bovine endometrial mesenchymal stem cells. Results are presented as mediam with interquartile range. IL-1α: interleukin 1α, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, PTGS2: prostaglandin-endoperoxide synthase 2…………………………………………………………………….. 90

Figura 3 – Cytokine concentration of IFN-α (a), INF-γ (b), IL-13 (c) and IL-1α (d) on culture medium of bovine endometrial mesenchymal stem cells challenged or not with LPS, after 2, 6, 12 and 24 hours of exposition. Asterisk (* at IL-1α) represents differences (P< 0.05) between moments. C: control, T: treated. Results are

presented as mediam with interquartile rage……………….……. 91

Figura 4 – Cytokine concentration of IL-1F5 (a), IL-21 (b), MIP-1β (c) and TNF-α (d) on culture medium of bovine endometrial mesenchymal stem cells challenged or not with LPS, after 2, 6, 12 and 24 hours of exposition. Asterisk (* at TNF-α) represents differences (P< 0.05) between groups (control x treated). C: control, T: treated. Results are presented as mediam with interquartile rage. ……………………………………...……… 92

Figura 5- Prostaglandin Ex2 concentration on bovine endometrial mesenchymal stem cells challenged or not with LPS culture medium after 2 and 24 hours of exposition. Comparison was done between moments and groups. C: control, T: treated. Results are presented as median with interquartile range. Asterisk (*) represents differences (P< 0.05) between groups and different letters represents difference (P< 0.05) between moments…………………………………………………………. 93

SUMÁRIO

Página

RESUMO... xvi

ABSTRACT... xviii

1 INTRODUÇÃO.. 1

2 JUSTIFICATIVA 4

3 REVISÃO DE LITERATURA……... 6

3.1 Células-tronco 7

3.1.1 Células-tronco mesenquimais/progenitoras provenientes do endométrio.. 8

3.2 Isolamento de CTMsE 10

3.3 Caracterização do cultivo celular de CTMsE 11

3.3.1 Caracterização de antígenos de superfície e intracelulares de CTMsE 12

3.3.2 Identificação do potencial de diferenciação de CTMsE 14

3.3.3 Capacidade de auto-renovação 15

3.4 Avaliação cromossômica de CTMsE.. 15

3.5 Criopreservação de CTMsE... 16

3.6 Meio condicionado de CTMs e Proteômica 17

3.7 Imunomodulação das CTMs 19

4 OBJETIVOS ……………………... 22

4.1 Objetivos Gerais 23

4.2 Objetivos Específicos 23

5 HIPÓTESES……………………... 25

6 REFERÊNCIAS 27

CAPÍTULO 1

Artigo Científico publicado no periódico “Cell International Biology”: Bovine endometrial cells: a source of mesenchymal stem/progenitor cells.. 36

CAPÍTULO 2

Artigo Científico publicado no periódico “Veterinary Immunology and Immunopathology”: Shotgun proteomic analysis of the secretome of bovine endometrial mesenchymal progenitor/stem cells challenged or not with bacterial lipopolysaccharide..……... 59

CAPÍTULO 3

Artigo Científico nas normas a ser submetido no periódico “Cytokine”: Evaluation of the immunomodulatory properties of bovine endometrial mesenchymal stem cells ………………………..……………..…………….. 79

CAPÍTULO 4

7 CONSIDERAÇÕES GERAIS 100

ANEXOS 102

MAIA, C.N.M. **Estudo comparativo do perfil imunofenotípico, potencial de diferenciação, capacidade de produção de citocinas e criopreservação de células estromais do endométrio de vacas durante o ciclo estral.** Botucatu, 2017. 150p. Defesa (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

RESUMO

As células-tronco de origem mesenquimal provenientes do tecido endometrial (CTMsE) e seu meio condicionado (MC) apresentam propriedades terapêuticas, e são alternativas promissoras para estudos na medicina veterinária. Estudos envolvendo CTMsE e seu MC ainda são considerados escassos na espécie bovina até o presente momento. Desta forma, o objetivo deste trabalho foi avaliar o potencial de diferenciação, perfil imunofenotípico, estabilidade cromossômica, eficiência de clonicidade, resposta à criopreservação das CTMsE de bovinos coletadas em duas fases do ciclo estral. Adicionalmente, avaliar o secretoma, produção de citocinas e de prostaglandina E_2 (PGE_2) das CTMsE estimuladas ou não com lipopolissacarídeo (LPS) bacteriano. Para tanto, foi colhido o útero de fêmeas hígidas (Fase II n=6/ Fase III n=6) para o isolamento das CTMsE por digestão enzimática. CTMsE em primeira passagem foram avaliadas quanto ao número de cromossomos e as em segunda passagem foi conduzido o ensaio de clonicidade. As CTMsE em terceira passagem (P3) foram submetidas a diferenciação nas linhagens adipogênica, condrogênica e osteogênica e caracterizadas em relação ao perfil imunofenotípico por citometria de fluxo (CF) (vimentina, CD29, CD44, MHC-II, CD34) e imunocitoquímica (vimentina e CD44). Adicionalmente, as CTMsE em P3 foram criopreservadas utilizando-se dois meios de criopreservação e avaliadas por CF antes e após a criopreservação. Para avaliação da produção de citocinas, PGE_2 e análise do secretoma, o MC das CTMsE foi colhido após 2, 6, 12 e 24 horas de desafio (grupo tratado - GT) ou não (grupo controle - GC) com LPS bacteriano. As proteínas identificadas foram classificadas de acordo com os processos biológicos, função molecular, componente celular e classe proteica. De acordo com os resultados foi observado uma população celular homogênea, com

morfologia fibroblastóide e aderente ao plástico. Na análise por CF as CTMsE expressaram elevada marcação para CD29, CD44 e vimentina, baixa marcação para CD34 e ausência de expressão para o marcador MHC-II. Ainda, apresentaram estabilidade cromossômica, alta eficiência de clonicidade e diferenças ($P > 0.05$) na produção de citocinas e PGE_2 após desafio ou não com LPS. Diferenças não foram observadas ($P > 0.05$) entre os meios ou fase após o descongelamento. Foram identificados 397 grupos de proteínas no GT e 302 no GC. Houve um enriquecimento positivo para proteínas relacionadas à resposta antibacteriana, ativação dos macrófagos, atividade de hidrolase e enzimas inibitórias no GT, e moléculas de atividade estrutural e filamentos intermediários no GC. Pode-se concluir que as CTMsE de bovinos apresentam nas condições experimentais clonicidade, multipotencialidade, estabilidade cromossômica e satisfatória resposta à criopreservação, o que corrobora para o estabelecimento de bancos de células para uso terapêutico ou novos estudos *in vitro*. Adicionalmente, tais células respondem ao LPS na concentração utilizada, por meio da produção de citocinas, podendo este modelo ser utilizado para avaliação da resposta inflamatória. Ainda, a secreção de proteínas principalmente relacionadas ao remodelamento, resposta imune e angiogênese fazem destas células e de seus meios, promissores para futura aplicação na terapia celular.

Palavras-chave: bovinos, caracterização, CTMsE, LPS, útero

MAIA, C.N.M. C**omparative study of the immunophenotypic profile, differentiation potential, capacity of production of cytokines and cryopreservation of endometrial stromal cells of cattle during the oestrous cycle.** Botucatu, 2017. 150p. Thesis (Doctorate) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, São Paulo State University.

ABSTRACT

Mesenchymal stem cells from the endometrial tissue (MSCsE) and their conditioned medium (CM) have important therapeutic properties and are alternatives for studies in veterinary medicine. Studies involving MSCsE and its CM are still considered scarce in the bovine species until now. Thus, the aim of this study was to evaluate evaluate the potential of differentiation, immunophenotypic profile, chromosomal stability, clonicity efficiency and cryopreservation response of MSCsE from bovines collected in two phases of the estrous cycle. Additionally, to evaluate the secretoma production of cytokine and prostaglandin E2 (PGE2) of MSCsE stimulated or not with bacterial lipopolysaccharide (LPS). For this, the uterus of healthy females (Phase II n = 6 / Phase III n = 6) was collected for isolation of MSCsE by enzymatic digestion. MSCsE in first passage were evaluated for the number of chromosomes and the second passage was conducted the clonicity assay. The MSCsE in third passage (P3) were differentiated into the adipogenic, chondrogenic and osteogenic lineages and characterized by the immunophenotypic profile by flow cytometry (FC) (vimentin, CD29, CD44, MHC-II, CD34) and immunocytochemistry (vimentin, CD44). Additionally, MSCsE in P3 were cryopreserved using two cryopreservation medium: and evaluated by FC before and after cryopreservation. For evaluation of cytokine, PGE_2 production and analysis of the secretome, CM was collected after 2, 6, 12 and 24 hours of challenge (treated group - TG) or not (control group - CG) with LPS. The identified proteins were classified according to the biological processes, molecular function, cellular component and protein class. According to the results, was observed a homogeneous cell population, with a fibroblastoid

morphology and adherent to the plastic. In FC analysis, the MSCsE expressed high labeling for CD29, CD44 and vimentin, low labeling for CD34 and absence of expression for the MHC-II marker. Still, they presented chromosomal stability, high efficiency of clonicity and differences ($P<0.05$) in the production of cytokines and PGE_2 after stimulus or not with LPS. Differences were not observed ($P>0.05$) between the medium or phase after thawing. 397 groups of proteins were identified in GT and 302 in CG. There was a positive enrichment for proteins related to antibacterial response, macrophages activation, hydrolase activity and inhibitory enzymes in TG, and structural activity molecules and intermediate filaments in CG. It is possible to conclude that bovine MSCsE present in the experimental conditions clonicity, multipotentiality, chromosomal stability and satisfactory cryopreservation response, which corroborates for the establishment of cell banks for therapeutic use or new in vitro studies. Additionally, these cells respond to LPS at the concentration used, by the production of cytokines, and this model can be used to evaluate the inflammatory response.

Key words: bovines, characterization, LPS, MSCsE, uterus

INTRODUÇÃO

1. INTRODUÇÃO

A terapia com células-tronco (CT) apresenta-se como uma prática crescente na medicina veterinária. O interesse nestas células dá-se devido as suas propriedades pró-regenerativas e imunomoduladoras (PAUL; ANISIMOV, 2013). Desta forma, tais células e também o meio em que elas são cultivadas tem apontado como uma alternativa promissora para a terapia, com possível uso em diversas enfermidades, incluindo as que atingem o sistema reprodutivo.

Os bovinos são considerados modelos experimentais e apresentam várias vantagens para serem utilizados em estudos clínicos com células-tronco de origem mesenquimal (CTMs) e extrapolação dos resultados na medicina humana (BOSNAKOVSKI et al., 2004). Dentre estas a facilidade de obtenção de tecido para isolamento, cultivo, expansão e caracterização *in vitro.* Adicionalmente, a quantidade amostral obtida em tais estudos permite o estudo *in vitro* da formação de bancos de células para utilização futura.

Em bovinos, há descrição do isolamento e cultivo de CTMs de diversas fontes tais como do cordão umbilical (XIONG et al., 2014), sangue do cordão umbilical (RAOUFI et al., 2011), fluido amninótico (ROSSI et al., 2014), derme (SUN et al., 2014), medula óssea (BOSNAKOVSKI et al., 2004), tecido adiposo (LU et al.,2014), glândula mamária (CHOUDHARY, 2014) e tecido endometrial (DONOFRIO et al., 2008).

O tecido endometrial é um tecido altamente regenerativo, composto de uma porção luminal, células epiteliais, estromais, endoteliais, fibras musculares e leucócitos. As CTMs endometriais (CTMsE) são células dinâmicas com a capacidade de crescimento e diferenciação durante o ciclo estral e gestação (DONOFRIO et al., 2008). Tais células, presentes no endométrio adulto de humanos e camundongos, normalmente se diferenciam em células endometriais estromais sob influência de fatores de crescimento e esteroides ovarianos (GARGETT et al., 2008).

As CTMs apresentaram resultados favoráveis em aplicações em degeneração endometrial em humanos (MENG et al., 2007), endometriose (MAMBELLI et al., 2013) e fibrose (ALVARENGA et al., 2016) em equinos. Sabe-se que em humanos, as CTMsE foram capazes de reconstruir um endométrio funcional em modelos de xenoenxerto de endometriose em ratos (MASUDA et

al., 2007), e foram descritas em estudos com modelos de doenças metabólicas (SANTAMARIA et al., 2011), cardíacas (BOCKERIA et al., 2013), e neurológicas (WOLLF et al., 2011) evidenciando seu potencial uso em terapias diversas. Entretanto, apesar dos bovinos serem utilizados como modelo experimental de afecções tanto *in vivo* quanto *in vitro*, há escassez de estudos referentes a biologia básica das CTMsE.

JUSTIFICATIVA

2. JUSTIFICATIVA

Estudos envolvendo a terapia celular vem surgindo, com o intuito de aumentar o conhecimento sobre o uso de CTMs de diversas fontes. Os bovinos podem ser utilizados como modelo experimental e a utilização do tecido endometrial para isolamento e obtenção de células estromais progenitoras mostra-se cada vez mais promissora para aplicação clínica e estudos *in vitro*. Entretanto, apesar dos grandes avanços na medicina humana, pouco é conhecido sobre o comportamento dessas células na espécie bovina, no que diz respeito à caracterização, composição do secretoma, atividade imunomoduladora e resistência a criopreservação. Tais conhecimentos são importantes para a aplicação terapêutica especialmente em enfermidades reprodutivas.

REVISÃO DE LITERATURA

3. REVISÃO DE LITERATURA

3.1. Células -tronco

As CTs podem ser definidas como células indiferenciadas com capacidade proliferativa e de autorenovação (WAGERS; WEISSMAN, 2004). Ademais, são capazes de responder a estímulos externos e dar origem a diferentes linhagens celulares mais especializadas (PEREIRA, 2008). Também são responsáveis pelo crescimento, reparação e homeostase de diversos tecidos, por mecanismos intrínsecos (fatores de transcrição) e extrínsecos (fatores de crescimento, interação entre as células e influências externas) (DU; TAYLOR, 2010).

As CTs podem ser classificadas de acordo com a origem tecidual ou quanto ao potencial de diferenciação, conforme demonstrado na Figura 1.

FIGURA 1: Classificação das células-tronco com relação à origem e potencial de diferenciação. Fonte: arquivo pessoal

Quanto a origem tecidual, as CTs podem ser classificadas em células embrionárias ou adultas. A primeira é derivada da massa celular interna e tem a habilidade de produzir as três camadas embrionárias germinativas. Já as adultas encontram-se presentes em diversos tecidos e tem a capacidade de diferenciar em pelo menos uma linhagem (Revisado por Rostamzadeh et al., 2015) e compreendem as células de origem hematopoiéticas, mesenquimais, epidermais, neuronais, dentre outras (AGOSTINI et al., 2013).

Quanto ao potencial de diferenciação, as CTs podem ser totipotentes, pluripotentes ou multipotentes. As células totipotentes referem-se às células do zigoto e pode dar origem a um indivíduo completo, incluindo seus anexos fetais. As pluripotentes correspondem às células de origem da massa celular interna do blastocisto e podem dar origem a quase todos os tecidos (exceto os extra-embrionários). As multipotentes são células com a capacidade de auto-renovação e podem gerar células comumente com a mesma origem embriológica, como as provenientes da medula óssea (FRIEL et al., 2005).

3.1.1 Células-tronco mesenquimais/progenitoras provenientes do endométrio

As CTMs são células estromais multipotentes que possuem a capacidade de auto-renovação e são responsáveis pela manutenção tecidual (GARGETT et al., 2016). Neste grupo de células estão incluídas as CTMs do tecido endometrial (CTMsE).

Em geral, as CTMsE apresentam-se como uma fonte de CTMs multipotentes adultas e com capacidade regenerativa (GHOBADI et al., 2015). Segundo a literatura as CTMs já foram isoladas de endométrio de mulheres (GARGETT et al., 2009, 2016), camundongos (CHAN; GARGETT, 2006), suínos (MIERNIK; KARASINSKI, 2012), bovinos (ŁUPICKA et al., 2015) e ovinos (LETOUZEY et al., 2015). Adicionalmente, estas células são capazes de se diferenciarem em diversas linhagens celulares sob estímulos específicos. Frente a estas características, acrescidas da fácil obtenção (PAL, 2015), essa fonte é considerada como uma nova e promissora alternativa para biologia reprodutiva e medicina regenerativa (GHOBADI et al., 2015). Atributos estes que tem despertado grande interesse para novas pesquisas tanto na medicina humana quanto veterinária.

A origem das CTs, particularmente no tecido endometrial ainda não é completamente elucidada. Todavia, acredita-se que possam estar relacionadas a três possibilidades: 1 – podem representar o epitélio fetal e CTMs as quais permanecem no endométrio adulto e se replicam; 2 – podem representar CTs provenientes de uma fonte hematogênica (tal como as da medula óssea) que

migram para o endométrio periodicamente ou frente a uma injúria ou 3 – uma combinação de ambos os fatores (Revisado por Morelli et al., 2012).

Diversos estudos em humanos suportam a segunda teoria de que a medula óssea daria origem às CTs presentes no estroma, epitélio e endotélio endometrial, confirmados por marcadores específicos dos doadores machos, encontrados no tecido endometrial de receptoras ratas (GARGETT et al., 2016). Recentemente, Łupicka et al. (2015) também comprovaram esta teoria em bovinos ao evidenciarem marcação positiva para o C-kit (marcador de célula hematopoética) no endotélio dos vasos linfáticos e sanguíneos no miométrio e principalmente estroma uterino. Essa marcação e também a expressão gênica foi observada tanto em animais jovens quanto idosos, o que sugere que a migração de CTs de origem medular ao útero não é idade dependente e ocorre durante toda a vida.

Em humanos, há evidências que as CTMsE podem ser responsáveis pela alta capacidade de regeneração do endométrio durante as fases reprodutivas (Revisado por Gargett et al.,2009) e uma população de células progenitoras/tronco presentes na porção basal do endométrio é capaz de dar origem a um tecido endometrial funcional, com a presença de glândulas, vasos e estroma (MASUDA et al., 2010). Neste contexto, em bovinos, o tecido endometrial também tem sido estudado como fonte de CTMs uma vez que é altamente regenerativo e contém células indiferenciadas, com alta plasticidade, capacidade de crescimento e diferenciação durante o ciclo estral e gestação (DONOFRIO et al., 2008, ŁUPICKA et al., 2015).

A aplicação terapêutica desta nova fonte de CTMs, ou seja, as CTMSE, já foi descrita em humanos em modelos de doenças cardíacas (BOCKERIA et al., 2013), neurológicas (WOLLF et al., 2011) e metabólicas (SANTAMARIA et al., 2011), e são propostas como alternativa no tratamento de regeneração endometrial (MENG et al., 2007). Até o presente momento, os estudos com CTMsE de bovinos estão ainda no início e portanto a aplicação clínica desta fonte ainda não foi relatada nesta espécie.

3.2 Isolamento de CTMsE

O útero é composto de três porções: o perimétrio, o miométrio e o endométrio. O endométrio apresenta uma parte funcional que se modifica com o estro ou gestação e uma parte basal, que permanece intacta nesses eventos e é responsável pela restauração da parte funcional (MARUYAMA et al., 2010; PRIEDKALNS; LEISER, 2006).

Em bovinos, a principal localização de CTMsE foi observada no estroma endometrial, apesar de também ter sido identificada em populações epiteliais e miometriais (ŁUPICKA et al., 2015).

Em animais domésticos, tais como os bovinos e suínos, as CTMsE podem ser colhidas do útero oriundo de abatedouro (MIERNIK; KARASINSKI, 2012; DONOFRIO et al., 2008, CABEZAS et al. 2014, ŁUPICKA et al., 2015) ou, como em ovinos, após histerectomia (LETOUZEY et al., 2015). Em humanos, a coleta pode ser realizada por histerectomia, biópsias ou curetagem (MASUDA et al., 2010).

Após colheita em abatedouro, para realização do isolamento celular, o útero pode ser dissecado longitudinalmente e feita a separação do tecido endometrial, do tecido miometrial. Após obtenção do tecido endometrial, o isolamento celular é feito por digestões enzimáticas com colagenase, DNAse, tripsina, dispase, usadas isoladamente (MIERNIK; KARASINSKI, 2012) ou em conjunto (DONOFRIO et al., 2008, CABEZAS et al. 2014, ŁUPICKA et al., 2015). Tal isolamento pode ser realizado com um ou dois processos de digestão, sendo que duas digestões visam uma melhor separação da fração epitelial da estromal (FORTIER et al. 1988).

Em cultivo celular, as CTMsE de bovinos crescem em monocamadas e apresentam morfologia fibroblastóide, além de boa aderência ao plástico, capacidade de diferenciação e de formação de colônias (DONOFRIO et al., 2008, CABEZAS et al. 2014). Tais características de cultivo celular também são compartilhadas com outras espécies tais como humanos (DIMITROV et a., 2008, MASUDA et al., 2010; GAAFAR et al., 2014) e suínos (SUBBARAO et al., 2015).

Apesar da utilização de métodos de separação das células estromais das epiteliais, é comum que o cultivo apresente contaminação com células epiteliais, que pode ser controlada com a troca de meio com 18 horas após

plaqueamento do cultivo primário (FORTIER et al., 1988), e também da tripsinização diferenciada (ANAND et al., 2012). Concomitantemente, o cultivo tende a se purificar com a realizações das passagens e ficar mais homogêneo (DIMITROV et al., 2008).

Em humanos, acredita-se que o cultivo de toda a população de células endometriais, ou seja, sem purificação em células estromais ou epiteliais, seja um pré-requisito para o cultivo e ativação da fração de *side population* (com propriedades de CT adultas). Também, que não apenas o contato entre as células, mas a produção de algum fator secretado pela população mista auxilia no processo de crescimento (MASUDA et al., 2010), evidenciando que uma pequena quantidade de células epiteliais no cultivo, assim como pode ser observado no cultivo de bovinos, não é prejudicial ao crescimento e isolamento celular.

3.3 Caracterização do cultivo celular de CTMsE

A Sociedade Internacional de Terapia Celular estabeleceu critérios mínimos para a caracterização das CTMs humanas os quais incluem: aderência ao plástico e morfologia fibroblastóide em cultivo, expressão dos receptores de superfície celular CD105, CD73 e CD90 (≥ 95% positivos) e ausência de expressão (≤ 2% positivos) dos marcadores CD14 ou CD11b, CD34, CD45, CD79α e MHC-II, além da demonstração *in vitro* para diferenciação osteogênica, adipogênica e condrogênica (DOMINICI et al., 2006).

Assim como em humanos, não há descrito um painel específico para a caracterização das CTMsE de bovinos. Desta forma, faz-se necessário que a identificação desse tipo celular no tecido endometrial seja de acordo com as propriedades funcionais de CTs que incluem características relacionadas a clonicidade, potencial de proliferação, capacidade de diferenciação em uma ou mais linhagens (GARGETT, 2007) e também a expressão de marcadores de superfície de origem mesenquimal (DIMITROV et al., 2008).

3.3.1 Caracterização de antígenos de superfície e intracelulares de CTMsE

Distintos antígenos tanto de superfície quanto intracelulares já foram descritos na literatura para a caracterização de CTMsE por citometria de fluxo e por reação em cadeia da polimerase (PCR). Entretanto, ainda há falta de um painel de marcadores específicos para melhor caracterização desta população celular.

Conforme pode ser evidenciado no Quadro 1, os marcadores descritos na literatura para as CTMsE podem ser utilizados para a separação das porções celulares por *sorting* (LETOUZEY et al., 2015), como também para caracterização das linhagens de origem mesenquimal (MORAES et al., 2016a, MIERNIK; KARASINSKI, 2012) ou hematopoiéticas (CABEZAS et al., 2014, MIERNIK; KARASINSKI, 2012) ou ainda para a detecção de células com pluripotência (CABEZAS et al., 2014, ŁUPICKA et al., 2015).

QUADRO 1: Painel de marcadores de superfície e intracelular já descritos em cultivos de CTMsE em diferentes espécies por citometria de fluxo ou PCR. (continua)

MARCADOR (CD)	ESPÉCIE	TÉCNICA	MARCAÇÃO (+ OU-)	AUTORES
CD34	Suínos	PCR	-	MIERNIK e KARASINSKI, 2012
		Citometria	-	SUBBARAO et al., 2015
	Bovinos	PCR	-	CABEZAS et al., 2014
		Citometria	-	MORAES et al., 2016a
CD45	Suínos	PCR	-	MIERNIK e KARASINSKI, 2012
		Citometria	-	SUBBARAO et al., 2015
CD9	Suínos	Citometria	-	SUBBARAO et al., 2015
OCT-4	Suínos	PCR	-	MIERNIK e KARASINSKI, 2012
			+	SUBBARAO et al., 2015
	Bovinos	PCR	+	CABEZAS et al., 2014
		Citometria/RT-PCR	+	ŁUPICKA et al., 2015

QUADRO 1: Painel de marcadores de superfície e intracelular já descritos em cultivos de CTMsE em diferentes espécies por citometria de fluxo ou PCR. (conclusão).

MARCADOR (CD)	ESPÉCIE	TÉCNICA	MARCAÇÃO (+ OU-)	AUTORES
CD44	Suínos	PCR	+	MIERNIK e KARASINSKI, 2012
		Citometria	+	SUBBARAO et al., 2015
	Bovinos	Citometria	+	MORAES et al., 2016 a
CD144	Suínos	RT-PCR	+	MIERNIK e KARASINSKI, 2012
CD105	Suínos	RT-PCR	+	MIERNIK e KARASINSKI, 2012
	Ovinos	Citometria	-	LETOUZEY et al., 2015
CD271	Ovinos	Citometria	+	LETOUZEY et al., 2015
PDGFRB	Ovinos	Citometria	-	LETOUZEY et al., 2015
CD90	Ovinos	Citometria	-	LETOUZEY et al., 2015
	Suínos	Citometria	+	SUBBARAO et al., 2015
CD146	Ovinos	Citometria	-	LETOUZEY et al., 2015
CD73	Ovinos	Citometria	-	LETOUZEY et al., 2015
SOX-2	Bovinos	PCR	+	CABEZAS et al., 2014
		Citometria	+	ŁUPICKA et al., 2015
	Suínos	PCR	+	SUBBARAO et al., 2015
NANOG	Bovinos	PCR	-	CABEZAS et al., 2014
		Citometria/PCR	+	ŁUPICKA et al., 2015
	Suínos	PCR	+	SUBBARAO et al., 2015
CD29	Suínos	RT-PCR	+	MIERNIK e KARASINSKI, 2012
		Citometria	+	SUBBARAO et al., 2015
	Bovinos	Citometria	+	MORAES et al., 2016a
VIMENTINA	Bovinos	Citometria	+	MORAES et al., 2016a
SOX-2	Bovinos	PCR	+	CABEZAS et al., 2014
		Citometria	+	ŁUPICKA et al., 2015
	Suínos	PCR	+	SUBBARAO et al., 2015
NANOG	Bovinos	PCR	-	CABEZAS et al., 2014
		Citometria/PCR	+	ŁUPICKA et al., 2015
	Suínos	PCR	+	SUBBARAO et al., 2015

3.3.2 Identificação do potencial de diferenciação de CTMsE

A diferenciação das CTMsE pode ser alcançada mediante a utilização de agentes indutores no cultivo celular (DIMITROV et al., 2008), sendo a diferenciação um dos critérios mínimos para a caracterização da linhagem celular. A possibilidade de diferenciação nas tri-linhagens já foi descrita para bovinos (ŁUPICKA et al., 2015), ovinos (LETOUZEY et al., 2015), humanos (GARGETT et al., 2009) e equinos (RINK et al., 2017). Adicionalmente, em suínos foi possível a diferenciação na linhagem adipogênica e osteogênica (SUBBARAO et al., 2015, MIERNIK; KARASINSKI, 2012). A resposta positiva à diferenciação nas tri-linhagens observada em CTMsE de bovinos mostra a alta plasticidade dessas células e a presença de CT indiferenciadas no útero (ŁUPICKA et al., 2015).

Após a exposição aos agentes indutores para diferenciação adipogênica, osteogênica ou condrogênica, pode ser realizada a coloração das células com *oil red, alizarim red* ou *von kossa* e *alcian blue*, respectivamente para avaliação da resposta à diferenciação. Em células induzidas à diferenciação adipogênica, osteogênica ou condrogênica pode-se observar a resposta mediante a identificação da presença de gotículas de lipídeos, depósitos de cálcio e mineralização da matrix extracelular (SUBBARAO et al., 2015) e coloração para identificação de glicoproteínas e glicosaminoglicanos, respectivamente (ŁUPICKA et al., 2015) além da alteração morfológica das células (DONOFRIO et al., 2008).

A resposta positiva à diferenciação também pode ser avaliada pela expressão de genes ou proteínas específicas como as de ligação de ácidos graxos, da lipoproteína lipase ou proteína ligadora dos adipócitos para a diferenciação adipogênica; do gene da osteonectina, biglican ou Runx2 (Runt relacionado ao fator de transcrição 2) para a diferenciação osteogênica (SUBBARAO et al., 2015) e dos genes do colágeno (colágenos tipo I, II ou X) para a diferenciação condrogênica (MERETOJA et al., 2013).

3.3.3 Capacidade de auto-renovação

Outra característica importante para a avaliação de CTMs é a capacidade de auto-renovação que pode ser avaliada pelo ensaio de unidades formadoras de colônias fibroblásticas (UFC-F).

Tal ensaio consiste no plaqueamento de um número reduzido de células (30-105 células/ cm^2) e após determinado tempo, que pode variar de 5 a 30 dias, (CABEZAS et al., 2014; MORAES et al., 2016a) procede-se com a coloração das colônias com cristal violeta a 1% em metanol e contagem das colônias coradas (MENSING et al., 2011). A eficiência de clonicidade é feita de acordo com a seguinte fórmula: (número de colônias/ número de células plaqueadas) x 100 (CABEZAS et al., 2014).

A clonicidade das CTMsE já foi avaliada e comprovada em bovinos (MORAES, et al., 2016a), ovinos (LETOUZEY et al., 2015), humanos (CHAN et al., 2004) e suínos (MIERNIK; KARASINSKI, 2012).

3.4 Avaliação cromossômica de CTMsE

A avaliação cromossômica é útil para o monitoramento da estabilidade genética de CTMs ao longo dos cultivos e também para a decisão da utilização das células futuramente (STULTZ et al., 2016) antes ou após a criopreservação.

Células que ficam muito tempo em cultivo podem ter as taxas de viabilidade reduzidas, e isso pode ser atribuído à instabilidade cromossômica e encurtamento do telômero, que culmina com a perda funcional de genes relacionados ao desenvolvimento celular (CUNHA et al., 2014).

A análise do cariótipo já foi realizada em CTMsE de bovinos, que tais células são cromossomicamente estáveis antes e após a criopreservação e, portanto, neste contexto seguras para a aplicação terapêutica (MORAES et al., 2016a).

3.5 Criopreservação de CTMsE

A criopreservação consiste na manutenção de CTMs à baixas temperaturas (-196ºC), levando a uma diminuição da atividade metabólica celular. As células criopreservadas podem ser utilizadas na medicina regenerativa, terapia celular ou para a formação de bancos de células (MARQUEZ-CURTIS et al., 2015). Apesar dos avanços já alcançados, pouco é conhecido sobre a criopreservação de CTMsE. Desta forma, o desafio do processo de criopreservação continua sendo a manutenção das características funcionais e morfológicas observadas nas células a fresco após o seu descongelamento.

Idealiza-se que as células deveriam ser criopreservadas mediante a redução gradativa da temperatura objetivando minimizar a formação de cristais de gelo intracelulares, uma vez que com a redução da temperatura a taxas ideais, há a formação de cristais de gelo extracelular que mudam a osmolaridade do meio e levam à desidratação equilibrada da célula (Revisado por Marquez-Curtis et al., 2015). O meio em que as células são criopreservadas é composto usualmente de um crioprotetor e aditivos, visando a redução dos danos causados as células durante o processo de criopreservação (Maia et al., 2017). O crioprotetor mais utilizado é o dimetilsulfóxido (DMSO), entretanto apresenta toxicidade em altas concentrações e pode ser nocivo às CTMs (FREIMARK et al., 2011; Revisado por Marquez-Curtis et al., 2015). O soro fetal bovino (SFB), também é utilizado nos meios de criopreservação, e participa da estabilização da membrana celular, ajuste osmótico, proteção contra os radicais livres de oxigênio (Revisado por Marquez-Curtis et al., 2015) e do processo de recristalização durante o descongelamento (RENZI et al., 2012).

Meios de criopreservação alternativos, com baixa concentração de SFB já foram descritos para criopreservação de CTMs de equinos. Os autores observaram que o MC (com 20% de SFB) não afetou as características biológicas das CTMs após criopreservação (MAIA et al., 2017), confirmando a importância da composição do meio de criopreservação para o crescimento celular após descongelamento e plaqueamento (RENZI et al., 2012).

3.6 Meio condicionado de CTMs e Proteômica

O meio condicionado (MC) refere-se ao meio em que as CTMs foram cultivadas em condições normais ou em situação de desafios (baixa tensão de oxigênio, privação de SFB). Estudos tem focado na aplicação clínica do MC devido a presença de fatores solúveis (fatores de crescimentos, inibidores de proteases) e/ou vesículas extracelulares em sua composição que foram secretados pelas células que apresentam propriedades terapêuticas.

As CTMs liberam fatores autócrino e parácrinos, conhecido como secretoma (SKALNIKOVA, 2013), o qual é considerado por alguns autores como o mais importante auxiliador dos efeitos reparativos direcionados às CTMs (PAUL; ANISIMOV, 2013). Tais fatores bioativos são liberados na forma solúvel ou em vesículas extracelulares, os quais participam da modulação da resposta imune mediada pelas CTMs, (LAVOIE; ROSU-MYLES, 2013) auxiliando na reparação tecidual (MAUMUS et al., 2013).

As proteínas estão envolvidas em diversos processos nos organismos vivos e possui um complexo mecanismo estrutural, de interações, dinâmica em concentração, degradação e/ou modificações que participam diretamente nos sistemas biológicos (MALMSTROM et al., 2007). As proteínas apresentam diversas características que não são previsíveis a partir de sequências gênicas ou níveis de transcrição, como por exemplo, as mudanças pós-transdução, as interações proteínas-proteínas e localizações subcelulares que afetam a função e atividade das proteínas (SUNG -MIN et al., 2010).

A proteômica pode ser definida como a técnica que avalia a proteína e seus sistemas de funções biológicos, considerando sua identificação, caracterização e quantificação em distintas amostras biológicas como órgãos, tecidos, fluidos biológicos, células (MALMSTROM et al., 2007) e o MC.

O proteoma pode ser definido como o conjunto de proteínas expressas em uma célula, tecido ou fluidos em determinado momento (HEIN et al., 2013), caracterizado em termos de quantidade, modificações pós-transducionais, interações e renovação. Esta análise permite a detecção de proteínas que sofrem modificações pós-tranducionais, tais como a maturação proteolítica, glicosilação ou fosforilação, todos esses processos que não podem ser observados em estudos genômicos. Isto é particularmente relevante, uma

vez que as vias de sinalização e de fosforilação de fatores de transcrição estão provavelmente envolvidas na auto-renovação e capacidade regenerativa das CTMs (ROCHE et al., 2006).

Para a identificação das proteínas presentes no secretoma, pode-se utilizar técnicas imunobiólogicas tais como ensaio imunoenzimático (ELISA), Western bloting ou RT-PCR (SKALNIKOVA, 2013) os quais identificam proteínas específicas e com funções biológicas conhecidas. A variação existente entre os resultados do secretoma de CTMS com estas técnicas é derivado da sensibilidade dos ensaios, meio de cultivo utilizado e origem das CTMs (LAVOIE; ROSU-MYLES, 2013).

Adicionalmente, as proteínas podem também ser analisadas de uma maneira mais global utilizando a espectrometria de massas acoplada a cromatografia líquida utilizando, por exemplo, a estratégia shotgun. Dessa forma, as amostras podem ser previamente digeridas e os peptídeos identificados e analisados qualitativamente e/ou quantitativamente (PALOMARES, 2014, SKALNIKOVA, 2013).

Nos últimos anos, na maioria das abordagens em proteômica clínica, a espectrometria de massa (MS) tem sido utilizada também para quantificar as proteínas presentes em amostras biológicas que estejam sob investigação. Recentemente a MS foi submetida a vários avanços técnicos em termos de sensibilidade e precisão, ampliando o número de diferentes abordagens que podem ser adotadas em estudos de proteômica (CAMERINIA & MAURIB, 2015).

O conhecimento da composição do secretoma de CTMs de diversas fontes é importante para o direcionamento das terapias com CTMs ou com seu MC e para avaliação do estudo do perfil proteico global, dos processos de proliferação, diferenciação, maturação e dos fatores parácrinos e autócrinos (STASTNA et al., 2009; BAIL, et al., 2012). O benefício da utilização do MC terapeuticamente já foi demonstrado em modelo experimental de esclerose múltipla, mostrando-se ser eficiente na recuperação dos modelos animais (BAIL, et al., 2012) e até apresentou efeitos similares ao tratamento com a própria CTM (SHIMOJIMA, et al., 2016). Também, o uso do MC apresentou efeitos positivos sobre a modulação de genes relacionados a inflamação em modelo experimental de inflamação uterina em éguas (LANGE-CONSIGLIO et al. 2015), além de

melhorar a taxa de proliferação de células endometriais em cultivo (CORRADETI et al., 2014).

3.7 Imunomodulação pelas CTMs

As CTMs exercem um grande papel de imunomodulação em diferentes tipos de células tais como em neutrófilos, monócitos, basófilos, células T e B. Embora tal processo ainda não seja completamente elucidado, sabe-se que tais efeitos se dão pela interação de contato entre células e também da secreção de fatores biologicamente ativos, fatores de crescimento, e citocinas (KYURKCHIEV et al., 2014).

As citocinas são pequenas proteínas secretada por diversas células, com efeito na comunicação e interação entre as células. Podem ser produzidas por linfócitos (linfocinas), monócitos (monicinas) e leucócitos (interleucinas-apresentando atividade em outro linfócito) e apresentarem atividade quimiotáticas (quimiocinas), na própria célula secretora (atividade autócrina), em células adjacentes (atividade parácrina) ou em células distantes (atividade endócrina) (ZHANG & AN, 2007). A definição das citocinas como pro ou anti-inflamatória ainda é controversa, uma vez que acredita-se que não exista uma única citocinas que esteja exclusivamente em uma única categoria (KYURKCHIEV et al., 2014). Elas podem ser ativadas por diversas outras citocinas e terem funções similares (ZHANG & AN, 2007).

Hoje é de conhecimento que os diferentes efeitos terapêuticos das CTMs são dependentes do microambiente em que estão, demonstrando que essas células não são espontaneamente imunossupressoras, necessitando de um estímulo para desencadear sua resposta que envolve uma complexa interação de diversos fatores (ABUMAREE et al., 2012).

Para exercerem seu papel, as CTMs podem ser ativadas por citocinas pro-inflamatórias (tais como TNF-α e INF-γ) ou pela ligação aos Toll-like Receptors (TLR) 3 ou 4, de produtos endógenos ou de patógenos (BERNARDO & FIBBE, 2013; ZACHAR et al., 2016). Após estímulo, as CTMs podem adquirir um fenótipo anti-inflamatório ou pro-inflamatório, e interagir com as células do sistema imune inato e adaptativo, produzindo moléculas imunossupressoras (Figura 2) (BERNARDO & FIBBE, 2013).

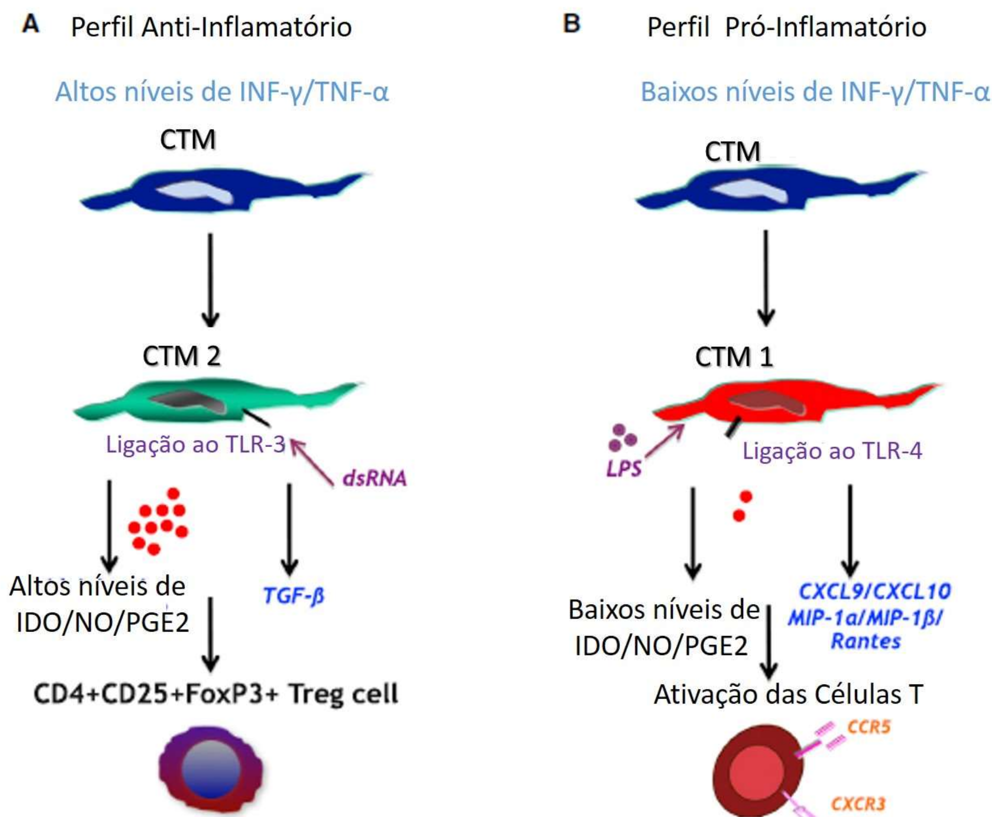


FIGURA 2: Modelo de ativação das MSCs por citocinas pro inflamatórias ou por TLRs. Ativação dos perfis anti-inflamatório (CTM 2) ou pró-inflamatórios (CTM 1). Adaptado de Bernardo & Fibbe, 2013. INF-γ: interferon γ; TNF-α: fator de necrose tumoral α; TNF-β: fator de necrose tumoral β; CTM: célula tronco mesenquimal; TLR: toll-like receptor; NO: óxido nítrico; IDO: indoleamina 2,3 dioxygenase; LPS: lipopolissacarídeo bacteriano; CYCL9; CXCL10; MIP-1α: proteína inflamatória de macrófago 1α; MIP-1β: proteína inflamatória de macrófago 1β.

Os fatores solúveis tais como prostaglandina E2 (PGE_2), IL-10 e fator estimulador de colônias de macrófagos (M-CFS) são alguns dos responsáveis pelos efeitos immunomodulatórios das CTMs, agindo na inibição da proliferação de linfócitos, diferenciação de monócitos em macrófagos e nas células dendritica. Tal secreção é iniciada após estímulo das células com fatores pró-inflamatório, ou lipopolissacarídeo bacteriano (ABUMAREE et al., 2012). A

PGE_2, é uma molécula imunossupressora, secretada após estímulo e exerce sua função mediando a supressão de linfócitos T, NK e macrófagos pelas CTMs (ABUMAREE et al., 2012). Aparentemente é o primeiro mediador responsável pela inibição da proliferação de células de defesa pelas CTMs (CARRADE & BORJESSON, 2013).

Frente a uma injúria tecidual, há a mobilização de células imunes, fibroblastos, células endoteliais e fatores biológico (citocinas, quimiocinas e fatores pró-inflamatórios) que entram em ação para a reparação e alcance da homeostase tecidual (SHI et al., 2012). Neste cenário, as CTMs são capazes de migrar ao sítio da lesão, se diferenciar em múltiplos tipos celulares, secretar citocinas e participar do processo de reparação e regeneração tecidual (CHEN et al., 2009).

OBJETIVOS

4. OBJETIVOS

4.1 Objetivo Geral

Isolar, cultivar, caracterizar e comparar amostras de células-tronco de origem mesenquimal obtidas a partir do tecido endometrial de vacas com corpos lúteos nas fases II e III do ciclo estral. Também, comparar dois meios de criopreservação de células-tronco de origem mesenquimal do tecido endometrial (CTMsE) de bovinos com a finalidade do futuro desenvolvimento de bancos de células para estudo *in vitro* e aplicação terapêutica. Adicionalmente, estudar a produção de citocinas e o secretoma das células estromais estimuladas ou não com lipopolissacarídeo bacteriano (LPS).

4.2 Objetivos Específicos

- Avaliar e comparar o padrão de isolamento e cultivo de células-tronco de origem mesenquimal do tecido endometrial de bovinos (úteros com ovários com corpo lúteo nas fases II e III).

- Avaliar e comparar a caracterização, capacidade de formação de colônias fibroblásticas, expressão de marcadores de superfície celular e intracelular (utilizando as técnicas de citometria de fluxo, imunocitoquímica e PCR), análise do cariótipo e potencial de diferenciação nas linhagens mesodermais das células-tronco de origem mesenquimal do tecido endometrial de bovinos (úteros com ovários com corpo lúteo nas fases II e III).

- Avaliar e comparar os efeitos da criopreservação com os diferentes meios de criopreservação sobre a viabilidade de células-tronco de origem mesenquimal do tecido endometrial de bovinos (úteros com ovários com corpo lúteo nas fases II e III) utilizando os marcadores iodeto de propídio e anexina após o descongelamento. Adicionalmente, determinar se o meio condicionado proporciona melhor viabilidade, reduz as taxas de apoptose e necrose, em comparação com o meio contendo alta proporção de SFB.

- Avaliar a produção de citocinas e de prostaglandina E_2 pelas células-tronco de origem mesenquimal do tecido endometrial de bovinos quando estimuladas pelo LPS bacteriano.

- Avaliar o secretoma das células estromais de origem mesenquimal estimuladas ou não com LPS bacteriano por espectrometria de massas acoplada a cromatografia liquida (nanoLC/MS/MS).

HIPÓTESES

5. HIPÓTESES

- As células-tronco de origem mesenquimal do tecido endometrial de bovinos (úteros com ovários com corpo lúteo nas fases II e III) apresentam perfil imunofenotípico, análise de cariótipo e potencial de diferenciação semelhantes porém com clonicidade distinta.

- As células-tronco de origem mesenquimal do tecido endometrial de bovinos (úteros com ovários com corpo lúteo nas fases II e III) apresentam taxas de viabilidade pós criopreservação satisfatórias e o meio de criopreservação contendo o meio condicionado confere melhor viabilidade e menor taxa de apoptose e necrose do que o meio contendo alta proporção de SFB, independente da fase luteal (II e III).

- As células-tronco de origem mesenquimal do tecido endometrial de bovinos apresentam a capacidade de produção de citocinas e prostaglandina E_2 *in vitro* sobre estímulo do LPS bacteriano.

- A composição proteica da secretoma das células-tronco de origem mesenquimal do tecido endometrial de bovinos (úteros com ovários com corpo lúteo nas fases II e III) estimuladas com LPS bacteriano é distinta daquelas não estimuladas, com a presença de proteínas com atividades antimicrobianas e imunomoduladoras.

REFERÊNCIAS

6. REFERÊNCIAS

ALVARENGA, M.A.; CARMO, M.T.; SEGABINAZZI, L.G.; GUASTALI, M.D.; MAIA, L.; LANDIM-ALVARENGA, F.C. Feasibility and safety of endometrial injection of autologous bone marrow mesenchymal stem cells in mare. **Journal of Equine Veterinaty Science**, v.42, p.12-18, 2016.

ANAND, V.; DOGRA, N.; SINGH. S.; KUMAR, S.N.; JENA, M.K.; MALAKAR, D.; DANG, A.K.; MISHRA, B.P.; MUKHOPADHYAY, T.K.; KAUSHIK, J.K.; MOHANTY, A.K. Establishment and characterization of a buffalo (Bubalus bubalis) mammary epithelial cell line. **Plos one**, v.7, n.7, p.1-14, 2012.

AGOSTINI, M., RUFINI, A., BAMPOTON, E.T.W., BERNASSOLA, F., MELINO, G., KNIGHT, R. The p53 Family and Stem Cell Biology. **IN: p53 in the Clinics**, p.65-7, 2013.

BAIL, L.; LENNON, D.P.; CAPLAN, A.I.; DECHANT, A.; HECKER, J.; KRANSO, J.; ZAREMBA, A.; MILLER, RH. Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. **Nature Neuroscience,** v.15, n.6, p.862-870, 2012.

BOCKERIA, L.; BOGIN, V.; BOCKERIA, O.; LE, T.; ALEKYAN, B.; WOODS, E.J.; BROWN, A.A.; ICHIM, T.E.; PATEL, A.N. Endometrial regenerative cells for treatment of heart failure: a new stem cell enters the clinic. **Journal of Translacional Medicine,** v.11, n.56, p.1-8, 2013.

BOSNAKOVSKI, D.; MIZUNO, M.; KIM, G.; ISHIGURO, T.; OKUMURA, M.; IWANAGA, T.; KODOSAWA, T.; FUJINAGA, T. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. **Experimental Hematology**, v.32, n.5, p.502–509, 2004.

CABEZAS, J.; LARA, E.; PACHA, P.; ROJAS, D.; VERAGUAS, D.; SARAVIA, F.; RODRÍGUEZ-ALVAREZ, L.; CASTRO F.O. The endometium of cycling cows contains populations of putative mesenchymal progenitor cells. **Reproduction of Domestic Animals**, v.49, p.550-559, 2014.

CAMERINI, S.; MAURI, P. The role of protein and peptide separation before mass spectrometry analysis in clinical proteomics. **Journal of Chromatography A**, v.1381, p.1-12, 2015.

CHAN, R.W.; GARGETT, C.E. Identification of label-retaining cells in mouse endometrium. **Stem Cells**, v.24, p.1529–1538, 2006.

CHOUDHARY, R.K. Mammary Stem Cells: Expansion and animal productivity. **Journal of Animal Science and Biotechnology,** v.5, n.36, p.5-36, 2014.

CORRADETTI, B.; CORREANI, A.; ROMALDINI, A.; MARINI, M.G.; BIZZARO, D.; PERRINI, C.; CREMONESI, F.; LANGE-CONSIGLIO, A. Amniotic membrane-derived mesenchymal cells and their conditioned media: potential candidates for uterine regenerative therapy in the horse. **Plos One**, v.9, n.10, p.1-9, 2014.

DIMITROV, R.; TIMEVA, T.; KYURKCHIEV, D.; STAMENOVA, M.; SHTEREV, A.; KOSTOVA, P.; ZLATKOV, V.; KEHAYOV, I.; KYURKCHIEV, S. Characterization of clonogenic stromal cells isolated from human endometrium. **Reproduction**, v. 135, n.4, p.551-558, 2008.

DOMINICI, M.; LE BLANC, K.; MUELLER, I.; SLAPER-CORTENBACH, I.; MARINI, F.C.; KRAUSES, D.S.; DEANS, R.J.; KEATINGS, A.; PROCKOP, D.J.; HORWITZ, E.M. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. **Cytotherapy**, v.8, n.4, p.315-317, 2006.

DONOFRIO, G.; FRANCESCHI, V.; CAPOCEFALO, A.; CAVIRANI, S.; SHELDON, I.M. Bovine endometrial stromal cells display osteogenic properties. **Reproductive Biology and Endocrinology**, v.6, n.65, p.1-9, 2008.

DU, H.; TAYLOR, H.S. Stem cells and reproduction. **Current Opinion in Obstetrics and Gynecology**, v.22, n.3, p.235–241, 2010.

FORTIER, M.A.; GUILBALT, L.A.; GRASSO, F. Specific properties of epithelial and stromal cells from the endometrium of cows. **Journal of Reproduction and Fertility**, v.183, n.1, p.239-248, 1988.

FRIEL, R.; VAN DER SAR, S.; MEE, P.J. Embryonic stem cells: understanding their history, cell biology and signaling. **Advanced Drug Delivery Reviews**, v.57, n.13, p.1894-1903, 2005.

GAAFAR, T.; OSMAN, O.; OSMAM, A.; ATTIA, W.; HAMZA, H.; HAWARY, R.E. Gene expression profiling of endometrium versus bone marrow-derived mesenchymal stem cells: upregulation of cytokine genes. **Molecular and Cellular Biochemistry**, v.395, n.1-2, p.29-43, 2014.

GARGETT, CE. Uterine stem cells: what is the evidence?" **Human Reproduction Update**, v.13, n1, p.87–101, 2007.

GARGETT, C.E.; CHAN, R.W.; SCHAWAB, K.E. Hormone and growth factor signaling in endometrial renewal: Role of stem/progenitor cells. **Molecular and Cellular Endocrinology**, v.288, n.1-2, p.22-9, 2008.

GARGETT, C.E.; SCHAWAB, K.E.; ZILLWOOD, R.M.; NGUYEN, H.P.T.; WU, D. Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. **Biology of Reproduction**, v.80, n.6, p.1136-1145, 2009.

GARGETT, C.E.; SCHWAB, K.E.; DEANE, J.A. Endometrial stem/progenitor cells: the first 10 years. **Human Reproduction Updade**, v.22, n2, p.137-165, 2016.

GHOBADI, F.; MEHRABANI, D.; MEHRABANI, G. Regenerative potential of endometrial stem cells: a mini review**. World Journal of Plastic Surgery**, v.4, n.1, p.3-8, 2015.

HEIN, M. Y. et al. Proteomic Analysis of Cellular Systems. In: Walhout, A. J. M., Vidal, M., et al. **Handbook of Systems Biology**. San Diego: Academic Press, 2013, p.3-25.

CONSIGLIO, A.; PERRINI, C.; ESPOSTI, P.; DERIGIBUS, M.C.; CAMUSSI, G.; PASCUCCI, L.; MARINI, M.G.; CORRADETTI, B.; BIZARRO, D.; CREMONESI, F. Effects of microvesicles secreted from equine amniotic-derived progenitor cells on in vitro lipopolysaccharide-treated tendon and endometrial cells. **Reproduction Fertility and Development**, v.28, p.244-245, 2015.

LAVOIE, J.R.; ROSU-MYLES, M. Uncovering the secretes of mesenchymal stem cells. **Biochimie**, v.95, n.12, p.2212-2221, 2013.

LETOUZEY, V.; TAN, K.S.; DEANE, J.A.; ULRICH, D.; GURUNG, S.; ONG, Y.R.; GARGETT, C.E. Isolation and characterization of mesenchymal stem/ stromal cells in the ovine endometrium. **PLoS ONE**; v.10, n.5, p.1-17, 2015.

LU, T.; XIONG, H.; WANG, K.; WANG, S.; MA, Y.; GUAN. Isolation and characterization of adipose-derived mesenchymal stem cells (ADSCs) from Cattle. **Applied Biochemistry and Biotechnology**, v.174, n.2, p.716-728, 2014.

ŁUPICKA, M.; BODEK, G.; SHPIGEL, N.; ELNEKAVE, E.; KORZEKWA, A.J. Identification of pluripotent cells in bovine uterus: in situ and in vitro studies. **Reproduction**, v.149, n.4, p.317-327, 2015.

MAIA, L.; CAMARGOS, M.D.; MORAES, C.N.; DELLÁQUA, C.P.F.; MOTA, L.S.L.S.; SANTILONI, V.; LANDIM-ALVARENGA, F.C. Conditioned medium: a new alternative for cryopreservation of equine umbilical cord mesenchymal stem cells. **Cell Biology International**, v.41, p.239-248, 2017.

MALMSTROM, J.; LEE, H.; AEBERSOLD, R. Advances in proteomic workflows for systems biology. **Current Opinion in Biotechnology**, v.18, n.4, p.378–384, 2007.

MAMBELLI, L.I.; WINTER, G.H.Z.; KERKIS, A.; MALSCHUTZKY, E.; MATTOS, R.C.; KERKIS, I. A novel strategy of mesenchymal stem cells delivery in the uterus of mares with endometrosis. **Theriogenology**, v.79, p.744-750, 2013.

MARQUEZ-CURTIS, L.; JANOWSKA-WEICZOREK, A.; MCGANN, L.E.; ELLIOT, J.A.W. Mesenchymal stromal cells derived from various tissues: biological, clinical and cryopreservation aspects. **Cryobiology**, v.71, n.2, p.181-197, 2015.

MASUDA, H.; MARUYAMA, T.; YAMANE, J.; IWANAMI, A.; NAGASHIMA, T.; ONO, M.; MIYOSHI, H.; OKANO, H.J.; ITO, M.; TAMAOKI, N.; NOMURA, T.; OKANO, H.; MATSUZAKI, Y.; YOSHIMURA, Y. Noninvasive and real-time assesment of reconstructed functional human endometrium in NOD/SCID/γ_c^{null} immnuodeficient mice. **Proceedings of the National Academy of Sciences of the United States of America,** v.104, n.6 p.1925-1930, 2007.

MAUMUS, M.; JORGENSEN, C.; NOEL, D. Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: Role of secretome and exosomes. **Biochimie**, v.95, n.12, p.229-2234, 2013.

MENG, X.; ICHIM, T.E.; ZHONG, J.; ROGERS, A.; YIN, Z.; JACKSON, J.; WANG, H.; GE, W.; BOGIN, V.; CHAN, K.W.; THÉBAUD B, RIORDAN, N.H. Endometrial regenerative cells: a novel stem cell population. **Jounal of Translacional Medicine**, v.5, n.5, p.1-10, 2007.

MENSING, N.; GASSE, H.; HAMBRUCH, N.; HAEGER, J.; PFARRER C.; STASZYK, C. Isolation and characterization of multipotent mesenchymal stromal cells from the gingiva and the periodontal ligament of the horse. **BMC Veterinary Research**, v. 7, n.42, p.1-13, 2011.

MERETOJA, V.V.; DAHLIN, R.L.; WRIGHT, S.; KASPER, F.K.; MIKOS, A.G. The effect of hypoxia on the chondrogenic differentiation of co-cultured articular chondrocytes and mesenchymal stem cells in scaffolds. **Biomaterial**, v.34, n.17, p.4266-4273, 2013.

MIERNIK, K.; KARASINSKI. J. Porcine uterus contains a population of mesenchymal stem cells. **Reproduction**; v.143, n.2, p.203-209, 2012.

MORAES, C.N.; MAIA, L.; DIAS, M.C.; DELLÁQUA, C.P.F.; MOTA. L.S.L.S.; CHAPWANYA, A.; LANDIM-ALVARENGA, F. C.; OBA, E. Bovine endometrial cells: a source of mesenchymal stem/progenitor cells. **Cell Biology Internationa**l, v.40, p.1332-1339, 2016a.

MORELLI, S.S.; YI, P.; GOLDSMITH, L.T. Endometrial Stem Cells and Reproduction. **Obstetrics and Gynecology International**, v.12, p.1-5, 2012.

PAL, L. Uterine stem cells – promise and possibilities. **Maturitas**, v.82, n.3, p.282-283, 2015.

PAUL, G.; ANISIMOV, S.V. The secretome of mesenchymal stem cell: Potential implications for neuroregeneration. **Biochimie**, v.95, p.2246-2256, 2013.

PALOMARES, G.P. Cromatografía. Análisis y separación de péptidos y proteínas. In: CORRALES, F., CALVETE, J.J. **Manual de Proteómica,** p. 33-55, 2014.

PEREIRA, L.V. A importância do uso das células tronco para a saúde pública. **Revista Ciência & Saúde Coletiva**, v.13, n.1, p.7-14, 2008.

PRIEDKALNS, J.; LEISER, R. Female Reproductive System. In: EURELL,J.A., FRAPPIER, B.L **Dellmann's textbook of veterinary histology.** Austrália, 2006, 256-279.

RAOUFI, M.F.; TAJIK, P.; DEHGHAN, M.M.; EINI, F.; BARIN, A. Isolation and differentiation of mesenchymal stem cells from bovine umbilical cord blood. **Reproduction of Domestic Animals**, v.46, n.1, p. 95-99, 2011.

RENZI, S.; LOMBARDO, T.; DOTTI, S.; DESSI, S.S.; DE BLASIO, P.; FERRARI, M. Mesenchymal stromal cell cryopreservation. **Biopreservation and Biobanking**, v.10, n.3, p.276-281, 2012.

RINK, B.E.; AMILON, K.R.; ESTEVES, C.L.; FRENCH, H.M.; WATSON, E.; AURICH, C.; DONADEU, F.X. Isolation and characterization of equine endometrial

mesenchymal stromal cells. **Stem Cell Research and Therapy**, v.8, p.1 66- 178, 2017.

ROCHE, S.; PROVANSAL, M.; TIRS, L.; JORGENSEN, C.; LEHMANN, S. Proteomics of primary mesenchymal stem cells. **Regenerative Medicine**, v.1, n.4, p.511-517, 2006.

ROSSI, B.; MERLO, B.; COLLEONI, S.; IACONO, E.; TAZZARI, P.L.; RICCI, F.; LAZZARI, G.; GALLI, C. Isolation and in vitro characterization of bovine amniotic fluid derived stem cells at different trimesters of pregnancy. **Stem Cell Reviews and Reports**, v.10, n.5, p.712-724, 2014.

ROSTAMZADEH, A.; ANJOMSHOA, M.; KURD, S.; CHAI, J.; JAHANGIRI, F.; NILFOROUSHZADEH M.A.; ZARE, S. The role of Wharton´s Jelly mesenchymal stem cells in skin reconstructive. **Journal of Skin Stem Cells,** v.2, n.2, 2015.

SUBBARAO, R.B.; ULLAH, I.; KIM, E.J.; JANG, S.J.; LEE, W.J.; JEON, R.H.; KANG, D.; LEE, S.L.; PARK, B.W.; RHO, G.J. Characterization and evaluation of neuronal trans-differentiation with electrophysiological properties of mesenchymal stem cells isolated from porcine endometrium. **International Journal of Molecular Sciences**, v.16, n.5, p.10934-10951, 2015.

SANTAMARIA, X.; MASSASA, E.E.; FENG, Y.; WOLFF, E.; TAYLOR, H.S. Derivation of insulin producing cells from human endometrial stromal stem cells and use in the treatment of murine diabetes. **Molecular Therapy**, v.19, n.11, p.2065-2071, 2011.

SHIMOJIMA, C.; TAKEUCHI, H.; JIN, S.; PARAJULI, B.; HATTORI, H.; SUZUMURA, A.; HIBI, H.; UEDA, M.; YAMAMOTO, A. Conditioned medium from the stem cells of human exfoliated deciduous teeth ameliorates experimental autoimune encephalomyelitis. **Journal of Immunology**, v.196, n.10, p.4164-4171, 2016.

SKALNIKOVA, H.K. Proteomic techniques for characterization of mesenchymal stem cell secretome. Biochimie, v.95, p.2196-211, 2013.

STASTNA, M.; ABRAHAM, M.R.; VAN EYK, J.E. Cardiac stem/progenitor cells, secreted proteins, and proteomics. **FEBS Letters,** v.583, p.1800-18007, 2009.

STULTZ, B.G.; McGINNIS. K.; THOMPSON, E.E.; LO SURDO, J.L.; BAUER, S,R.; HURSH, A. Chromosomal stability of mesenchymal stromal cells during in vitro culture. **Cytotherapy**, v.18, n.3, p.336-343, 2016.

SUNG-MIN, A.; SIMPSON, R.; LEE, B. Genomics and proteomics in stem cell research: the road ahead. **Anatomy and Cell Biology**, v.43, p.1-14, 2010.

SUN, T.; YU, C.; GAO, Y.; ZHAO, C.; HUA, J.; CAI, L.; GUAN, W.; MA, Y. Establishment and biological characterization of a dermal mesenchymal stem cells line from bovine. **Bioscience Reports**, v.34, n.2, p.139-146, 2014.

WAGERS, A.J.; WEISSMAN, I.L. Plasticity of adult stem cells. **Cell,** v.116, n.5, p.639-648, 2004.

WOLLF, E.F.; BING-GAO. X.; YAO, K.V.; ANDREWS, Z.B.; DU, H.; ELSWORTH, J.D.; TAYLOR, H.S. Endometrial stem cell transplantation restores dopamine production in a Parkinson's disease model. **Journal of Cellular and Molecular Medicine**, v.15, n.4, p.747-755, 2011.

XIONG, H.; BAI, C.; WU, S.; GAO, Y.; LU, T.; HU, Q.; GUAN, W.; MA, Y. Biological characterization of mesenchymal stem cells from bovine umbilical cord. **Animal Cells and Systems**, v.18, n.1, p.55-67, 2014.

CAPÍTULO 1

Manuscrito publicado no periódico“Cell International Biology”

Bovine endometrial cells: a source of mesenchymal stem/progenitor cells

Running head: Description and cryopreservation of bovine eMSCs

Carolina Nogueira de Moraes[a*], Leandro Maia[a], Marianne Camargos Dias[a], Camila P. Freitas DellÁqua[a], Ligia Souza Lima Silveira da Mota[b], Aspinas Chapwannya[c], Fernanda da Cruz Landim-Alvarenga[a], Eunice Oba[a]

[a] Department of Animal Reproduction and Radiology, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil

[b]Department of Genetics, São Paulo State University, Botucatu, São Paulo, Brazil

[c]Ross University School of Veterinary Medicine, Department of Clinical Sciences, Basseterre, St Kitts and Nevis

*Corresponding author:

carolnmoraes@hotmail.com

Address: Prof. Doutor Walter Mauricio Correa w/n, Botucatu/São Paulo/Brazil

Telephone/Fax number: 55(14) 3880-2119

Key word: cow, characterization, cryopreservation, endometrium, progenitor cells

Abbreviation list:

AN: Annexin V

CE: Cloning efficiency

CD: Cluster of differentiation

CFU-F: Fibroblastic colony-forming unit assay

CL: Corpus luteum

DMEM: Dulbecco′s Modified Eagle′s Medium

eMSCs: Endometrial mesenchymal stem/progenitor cells

FC: Flow cytometer

FITC: Fluorescein isothiocyanate

FBS: Fetal bovine serum

HBSS: Hank's Balanced Salt Solution

MHC: Major histocompatibility complex

MSCs: Mesenchymal stem/progenitor cells

PI: Propidium iodide

SEM: standard error of the mean

Abstract

Endometrial mesenchymal stem/progenitor cells (eMSCs) are multipotent cells known to modulate the immune system, and have clinical application for human and animal health. This makes these bovines cells attractive for dual use as cellular therapy and experimental model. The aim of this study was to isolate, evaluate the differentiation potential, immunophenotypic and immunocytochemistry characteristics, chromosomal stability, cloning efficiency and cryopreservation response of bovine eMSCs collected in two phases of the estrous cycle. For this, cells were isolated and submitted to differentiation for adipogenic and osteogenic lineage. The cells were then characterized by flow cytometer (FC) (vimentin, CD29, CD44, MHC-II, CD34) and immunocytochemistry (vimentin, pan-cytokeratin, CD44) and submitted to cytogenetic and cloning efficiency assay. The cells were also cryopreserved using two different medium of cryopreservation and analyzed by FC for viability, necrosis, late-apoptosis+necrosis and initial apoptosis rates before and after cryopreservation. We obtained homogeneous cell populations which have fibroblastic morphology and adherence to plastic. These cells expressed high levels of markers CD29, CD44 and vimentin, low expression levels for CD34 and no MHC-II. The cells were chromosomally stable (2n=60) with high cloning efficiency and no difference ($P>0.05$) between medium of cryopreservation or phase was observed after thawing. We showed the presence and differentiation potential of bovine eMSCs, with chromosomal stability and great response to cryopreservation with both medium, which has implications for build biobanks or development of new therapeutic approaches to combat uterine diseases or to study.

1. Introduction

Mesenchymal stem/progenitor cells (MSCs) are multipotent cells which have attracted great interest in the fields of human and animal medicine because of their unique immunomodulatory properties. Many studies have described the feasibility of these cells as therapeutics because of their antiapoptotic and self-renewal properties. Specifically, these cells are known to down-regulate immune responses by promoting regulatory T cells and inhibit cytotoxic T cell proliferation (Stenger et al, 2015).

Bovines are a suitable experimental model, and have several advantages for use in clinical studies on application of MSCs for human medicine (Bosnakovski et al., 2004). The cells can readily be obtained from endometrial tissue, and expanded in vitro. In addition, it is possible to acquire large tissue samples for biobanking cells with good viability for future use in regenerative medicine.

The endometrial tissue is a highly regenerative tissue and contains endometrial stromal cells which are dynamic, and have the capacity for growth and differentiation during the estrous cycle and pregnancy in cows (Donofrio et al., 2008). The presence of endometrial MSCs (eMSCs) have been described in other mammals such as human (Garget et al., 2015), pig (Miernik and Karasinski, 2012), ovine (Letouzey et al., 2015) and mouse (Chan and Gargett, 2006).

MSCs cells are a viable alternative in the treatment of endometrial degeneration in humans (Meng t al., 2007), endometrosis (Mambelli et al., 2013) and fibrosis (Alvarenga et al., 2016) in mares. In humans, eMSCs or menstrual blood MSCs were used in cardiac (Bockeria et al., 2013), neurological (Wollf et al., 2011) and models of metabolic diseases (Santamaria et al., 2011) showing their great application. In non-primate animals which do not undergo menstruation, eMSCs participate in endometrial remodeling after each estrus event, and also in the regeneration of endometrial stroma

during the postpartum period (Letouzey et al., 2015). Thus these cells may have a direct application in treatment of reproductive pathologies that require an endometrial remodeling such as endometritis and fibrosis. Only a few studies describe in detail the presence of these cells in bovines, and even fewer give an in–depth characterization of these cells, which would be of great interest in clinical treatments.

To our knowledge there is no study on chromosomal stability or potential of cryopreservation of bovine eMSCs with reinforce the possibility of the creation of biobanks for future therapeutic use. So, the purpose of this study was to isolate, culture and characterize MSCs obtained from the bovine endometrium using the techniques of flow cytometry, immunocytochemistry, cloning efficiency and cytogenetics. In addition, we evaluated the response to cryopreservation using two medium of cryopreservation. These cells were collected and compared at two phases of the estrous cycle, and our findings can contribute for a better knowledge of biological properties and therapeutic potential of bovine MSCs.

2. Material and Methods

The study was performed according to the ethical guidelines recommended by the National Council for Control of Animal Experimentation and the College of Animal Experimentation, and it was approved by the institution's Animal Care and Experimentation Ethics Committee (Protocol Number 152/2014).

2.1. Selection of endometrial material

The uteri and ovaries of non-pregnant cows (n = 12) were selected at a local slaughterhouse immediately after slaughter and kept on ice until processing in the laboratory. The physiological statuses of the tracts were determined by observation of

uterine and ovarian structures, particularly the corpus luteum (CL) according to methodology described by Ireland et al. (1980) and Chapwanya et al. (2013). All the animals selected were estrous cycling (Phase II; n = 6/ Phase III; n = 6), with no evidence of uterine disease.

2.2 Processing, isolation and culture of MSCs

The uterine horn ipsilateral to the CL corresponding to each phase was sectioned and kept in 70% alcohol solution for 2 minutes (Chapwanya et al., 2013). Subsequently, the endometrial tissue was separated from myometrial tissue with the aid of a sterile scissors, and washed with wash solution composed of HBSS solution (50 mL) supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL), amphotericin B (3µg /mL) (Thermo Fisher Scientific®, USA) and amikacin (22µg / mL) (Teuto®, BRA).

The endometrial tissue was cut into small pieces and submitted to digestion performed in two steps (Fortier et al. 1988 with modifications). The first digestion was done using 0.3% trypsin (Sigma®, USA) in solution of 10 mL of HBSS (Thermo Fisher Scientific®, USA) for 3 hours at 22°C under agitation. Subsequently, the tissue was washed with wash solution and filtered in 40 µm filter (Becton Dickinson and Company®, USA). The second digestion was done with a solution composed 0.5 mg/mL of trypsin, 0.5 mg/mL of collagenase, 1 mg/mL of bovine serum albumin and 0.1 mg/mL DNase I (Sigma®, USA) in HBSS (Thermo Fisher Scientific®, USA) for 1.5 hours/37°C. After filtration in filter 40 µm, the material was resuspended in HBSS medium with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific®, USA) and centrifuged (Hermle centrifuged Z200A, GER) twice at 100 x g/10 minutes.

After viability assay with Tripan blue solution 0,4% (Gibco®, USA), the material was plated (1.25 x 10^5 cell/ 24-well plate) and cultured in medium comprising

DMEM high glucose/F12 (1:1), 20% FBS, penicillin (100IU/mL), streptomycin (100 µg /mL), amphotericin B (3µg/mL) (Thermo Fisher Scientific®, USA) and amikacin (11µg/mL) (Teuto®, BRA) at 37.5°C in humid atmosphere containing 95% air and 5% CO_2. The first change of medium occurred 18 hours after to remove the epithelial cells (Fortier et al., 1988) and additionally the differential trypsinization (Anand et al., 2012) with 0.125% of trypsin at 0.05% (Sigma®, USA) was performed to aid in the purification of samples on subsequent passages.

2.3 Immunophenotypic analysis

Immunophenotypic analyses were performed on cells in the third passage (Phase II; n=5/ Phase III; n=5).

For the analysis, the following antibodies were used: mouse anti-vimentin (clone V9, MCA862, AbD Serotec-Rad®, UK), mouse anti–CD29:Alexa fluor 647 (clone TS2/16, 303007, BioLegend®, USA), mouse anti-bovine CD44:FITC (clone IL-A118, MCA2433F, AbD Serotec®, UK), mouse anti-horse MHC–II:FITC (clone CVS20, MCA1085F, AbD Serotec®, UK) and rabbit anti CD-34:FITC (polyclonal, orb247244, Biorbyt®, USA). For the antibodie mouse anti-vimentin cells were previously fixed and permeabilized with Cytofix/Cytoperm™ (Becton Dickinson and Company®, USA) and incubation with the secondary goat-anti mouse:FITC (ab7064, abcam®, USA). The acquisition was made by Flow LSR Fortessa equipment (BD Biosciences, USA) and the analysis was done using the BD FACSDiva™ software (BD Biosciences, USA). During the analysis were accounted for 10,000 events.

2.4 Immunocytochemistry characterization

Immunocytochemistry analysis was done in third passage (Phase II; n=3 / Phase; III n=3) on 24 well-plates (Sarstedt®, USA) according to methodology already described by our group (Maia et al., 2013). The antibodies tested were mouse-anti CD44 (1:100, clone BAG40A, WS0507B-100, VMRD®, USA), mouse anti-vimentin (1:200, clone V9, M0725, Dako®, UK) and mouse anti-pan-cytokeratin (1:100, clone AE1/AE3, M3515, Dako®, USA) with reactivity proven for the species for the last two (Souza et al., 2014). After detection and revelation, the evaluation of the reaction was performed in an inverted light microscope, with a digitizer (LeicaVR Microsystems®, GERM).

2.5 Assays for differentiation

The differentiation assay was done for adipogenic and osteogenic (Phase II; n=6 / Phase III; n=6) lineages with cells in third passage. For this, cells were plated in 6-wells (5200 cells/cm^2) or 24 wells (25000 cells/cm^2) plates for adipogenic and osteogenic differentiation, respectively. After 60% confluence, the differentiation medium was added to the subcultures in triplicate, according to manufacturer's recommendations (Thermo Fisher Scientific®, USA) added by 5% rabbit serum (Maia et al., 2013) or 20% FBS (Toupadakis et al. 2015) at adipogenic and osteogenic medium, respectively.

The confirmation of the osteogenic and adipogenic differentiation was performed, respectively, after 14 and 8 days by demonstrating the deposition of calcium matrix using the histological staining method Alizarin red pH 4.2 (Sigma1) and the presence of intracytoplasmic fat droplets using the dye Oil red 0.5% in alcohol isopropyl (Sigma®).

2.6 Cytogenetic analysis

Cytogenetic analysis was performed on samples (Phase II; n=6 / Phase III; n=6) at first passage using the synchronization technique of cellular cycle by the method of methotrexate / thymidine / Colchicine (0.0016%) (Maia et al., 2013).

2.7 Fibroblastic colony-forming unit assay (CFU-F) for determination of cloning efficiency (CE)

CFU-F assay (Phase II; n= 5/ Phase III; n= 5) was made with cells on second passage according to Messing et al. (2011) with modifications. For this, cells were seeded (105 cells/cm^2) in 6- wells plates, in triplicate. The exchange of medium was done with 72 hours and at the 5ºday cultures were fixed and stained with crystal-violet in 100% methanol. Colonies with more than 20 cells were accounted and the CE was done using the formula = (counted colonies/ cells seeded) x 100.

2.8 Cryopreservation

The bovine eMSCs from both phases were cryopreserved at a final concentration of $1x10^6$ cell/cryotube with the following medium of cryopreservation: Medium 1 (MI): 90% FBS (Thermo Fisher Scientific®, USA) + dimethyl sulfoxide (DMSO) (Sigma®, USA), 10µL/mL penicillin/streptomycin, 3µg/mL amphotericin B; Medium 2 (MII): 90% conditioned medium (DMEM high glucose/F12 (1:1), 20% FBS, penicillin (100IU/mL), streptomycin (100 µg/mL), amphotericin B (3µg/mL) (Thermo Fisher Scientific®, USA) and amikacin (11µg/mL) (Teuto®, BRA)) + 10% DMSO (Sigma®, USA).

Samples were cryopreserved at a controlled cryogenic container (Mr. Frosty, Nalgene, 5100-001), maintained on a -80ºC freezer (Thermo Fisher Scientific®, USA) for 24 hours and conserved on liquid nitrogen for 1 month. After this period, cells were

thawed and samples cryopreserved with MI and MII (Phase II and Phase III n= 5) were evaluated by FC.

The FC analyses was done before and after cryopreservation using annexin V APC (AN, Becton Dickinson® and Company, USA) and propidium iodide (PI, Sigma®, USA) for viability (PI- AN-), necrosis (PI+AN-), late-apoptosis +necrosis (PI+ AN+) and initial apoptosis (PI-AN+) (Ranera et al., 2012).

2.8 Data analysis

Data from differentiation assays and immunocytochemistry were presented descriptively and compared qualitatively during the phases (II and III). The other variables with normal distribution were analyzed by t-test or submitted to the non-parametric Mann-Whitney rank sum test at the SigmaPlot software (version 11.0, 2008), adopting $P<0.05$ as significant. The results were presented as mean and standard error of the mean (SEM).

3. Results and Discussion

At the present study we observed results very interesting and promising in bovine eMSCs independent of the phase of estrous cycle studied. These findings mainly include the high expression of stem/progenitor cells markers (CD29 and C44), low immunogenicity (no expression of MHC-II), chromosomal stability (2n=60) excellent clonicity, differentiation potential on mesodermal lineages, as well as excellent resistance to cryopreservation demonstrated by the good post thaw viability. These are important and desirable characteristics for the establishment of cells biobank with progenitor cells lines standardized for future use in cell therapy and/or further use for in vitro studies.

Here, the bovine eMSCs at the two estrous cycle phases (II and III) were adherent to plastic and had fibroblastoid morphology within six hours of culture. Immunophenotypic profile by FC of bovine eMSCs revealed high expression for CD29, CD44 and vimentin, low expression for CD34 and no expression for MHC-II, with no difference ($P>0.05$) between phases (Figure 1). We emphasize that the threshold used for negative expression was greater than 3%. Qualitative immunocytochemistry analysis of bovine eMSCs revealed positive staining for CD44, vimentin and pan-cytokeratin (Figure 2) with the same pattern of staining at both phases.

Unlike human, which counts with a panel of specific surface markers (Dominici et al., 2006), specific surface markers for bovine eMSCs are not well-known and much less established. The significant marking in both immunophenotypic and immunocytochemistry evaluation for CD44 and vimentin seen in our study were previously described in amniotic fluid-derived MSCs (Rossi et al., 2014). The CD44 and CD29 expression was reported in porcine eMSC (Miernik & Karasinski, 2012), bovine umbilical cord (Xiong et al., 2014) and human eMSCs (Gargett et al., 2009; Verdi et al.2014). The absence of marking for MHC-II here maybe due to low immunogenicity of the endometrial cells, with is of great interest for therapeutic use.

Differentiation assays of adipogenic and osteogenesis lineages of bovine eMSCs were confirmed in all samples with excellent response after staining (Figure 3). We reinforce the results of Łupicka et al. (2015a,b) which showed that uterine stromal cells differentiated to adipogenic, chondrogenic and osteogenic lineage upon specific condition, confirming the wide plasticity of these source. We reached great response to differentiation once Donofrio et al (2008) demonstrated the osteogenic potential of endometrial stromal cells and Cabezas et al. (2014) the osteogenic and chondrogenic potential of endometrial putative mesenchymal progenitor cells from late luteal phase. It

could be inferred that when bovine eMSCs are exposed to inducing agents, they have differing sensitivity and hence fail to differentiate in a too early luteal phase.

According to karyotype analysis based on 100% of metaphases, we showed by the first time, until our knowledge, that these bovine eMSCs have normal chromosome number (2n=60) (Figure 4B) and chromosomal stability also after cryopreservation (data not show). This shows that neither isolation technique nor culture or cryopreservation altered the genetic makeup of these cells. Additionally, CE revealed great capacity of clonicity from bovine eMSCs and the formation of delimitated colonies (Figure 4A) without difference ($P=0.750$) between phases. This CE rate is excellent and comparable to samples from umbilical cords or adipose tissue (Lu et al., 2014; Xiong et al., 2014). Therefore, creating cell biobanks for future use is quite feasible once eMSCs are stable and suitable for therapeutic use.

The cryopreservation using DMSO as a protective solution in combination with a progressive reduction of temperature is efficient in the preservation of good viability rates for bovine cells from amniotic fluid and umbilical cord, once enables the culture after thawed (Cunha et al., 2014). To our knowledge, this is the first report showing the possibility of cryopreservation of bovine eMSCs using two medium of cryopreservation at a controlled temperature rate, allowing the formation of cell biobank. Before cryopreservation cells from phase III presented a better viability rate ($P=0.01$) and lower proportion of cells on initial apoptosis ($P=0.04$). A decrease ($P=0.03$) on the viability rate after cryopreservation was detected on Phase III using both medium, comparing to fresh samples, and at both phases late apoptosis + necrosis increased ($P>0,05$) on cells cryopreserved with MI and MII comparing to fresh samples. No difference ($P>0.05$) was detected in any of the variables after cryopreservation comparing

the phases and medium used. Data before and after cryopreservation are presented at Table 1.

4. Conclusion

At the present work we showed for the first time at bovine eMSCs, until our knowledge, the chromosomal stability and potential of cryopreservation with two different mediums. We verified that bovine eMSCs derived from estral uteri adhere to plastic, have fibroblastoid morphology, good clonicity, differentiation potential and immunophenotypic progenitor/stem cells characteristics beside good viability rates after thawing at the two studied phases and with the two medium used. Thus bovine eMSCs can be used in further studies allowing the formation of biobanks and may have greater potential to impact clinical outcome because they are stable. Depending on the objective (therapeutic use, in vitro studies, formation of cell biobank), cells from both phases could be used, supposing be preferable work with fresh cells from phase III and with cryopreserved cells from phase II according to a possibly better answer and resistance to cryopreservation from this last one. Additionally, both medium used at the present work were suitable to maintain the quality of cells and we highlight the M2 with is commonly discarded and could be used for this propose. Pre-clinical studies of MSCs are still warranted to further establish the most ideal source of MSCs and eMSCs mechanism of action, particularly in potentiating tissue repair.

Funding:

Fundação de Amparo à Pesquisa do Estado de São Paulo (Proc.2014/20447-2, Proc. 2015/18964-1) for the scholarship and financial support (2015/01057-1).

5. References

1.Alvarenga MA, Carmo MT, Segabinazzi LG, Guastali MD, Maia L, Landim-Alvarenga FC (2016). Feasibility and safety of endometrial injection of autologous boné marrow mesenchymal stem cells in mare. J Equine Vet Sci;42;12-18.

2.Anand V, Dogra N, Singh S, Kumar SN, Jena MK, Malakar D, Dang AK, Mishra BP, Mukhopadhyay TK, Kaushik JK, Mohanty AK (2012). Establishment and Characterization of a Buffalo (Bubalus bubalis) Mammary Epithelial Cell Line. Plos one;7,1-14.

3. Bockeria L, Bogin V, Bockeria O, Alekyan B, Woods EJ, Brown AA, Ichim TE, Patel AN. (2013). Endometrial regenerative cells for treatment of heart failure: a new stem cell enters the clinic. J Transl Med;5:2-8.

4. Bosnakovski D, Mizuno M, Kim G, Ishiguro T, Okumura M, Iwanaga T, Kadosawa T, Fujinaga T. (2004). Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. Exp Hemat;32:502-509.

5. Cabezas J, Lara E, Pacha P, Rojas D, Veraguas D, Saravia F, Rodríguez-Alvarez L, Castro FO. (2014). The endometium of cycling cows contains populations of putative mesenchymal progenitor cells. Reprod Dom Anim;49:550-559.

6. Chan RW, Gargett CE (2006). Identification of label-retaining cells in mouse endometrium. Stem Cells;24:1529–1538

7. Chapwanya A, Meade KG, Doherty M, Callanan JJ, O´Farrelly C. (2013). Endometrial epithelial cells are potent producers of tracheal antimicrobial peptide and serum amyloid A3 gene expression in response to *E.coli* stimulation. Vet Immunol Immunopathol; 151: 157-162.

8. Cunha ER, Martins CF, Silva CG, Bessler HC, Báo SN. (2014). Effects of prolonged in vitro culture and cryopreservation on viability, DNA fragmentation, chromosome stability and ultrastructure of bovine cells from amminiotic fluid and umbilical cord. Reproduction in Domestic Animals;49:806-8012.

9. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy;8:315–7.

10. Donofrio G, Franceschi V, Capocefalo A, Cavirani S, Sheldon IM. (2008). Bovine endometrial stromal cells display osteogenic properties. Reprod Biol Endocrinol;6:1-9.

11. Fortier MA, Guilbault LA, Grasso F. (1988). Specific properties of epithelial and stromal cells from the endometrium of cows. J Reprod Fert;83:239-248.

12. Gargett CE, Schwab KE, Zilwood RM, Nguyen HPT, Wu D. (2009). Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. Biol Reprod; 80:1136-1145.

13. Garget CE, Schwab KE, Deane JA. (2015). Endometrial stem/progenitor cells: the first 10 years. Hum Reprod Updade; 0:1-27.

14. Ireland JJ, Murphee RL, Coulson PB. (1980). Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J Dairy Sci; 63:155-160.

15. Letouzey V, Tan KS, Deane JA, Ulrich D, Gurung S, Ong YR, Gargett CE. (2015). Isolation and characterization of mesenchymal stem/ stromal cells in the ovine endometrium. PLoS ONE; 10:1-17.

16. Lu T, Xiong H, Wang K, Wang S, Ma Y, Guan W. (2014). Isolation and characterization of adipose-derived mesenchymal stem cells (ADSCs) from cattle. Appl. Biochem. Biotechnol. 174, 717:728.

17. Łupicka M, Socha B, Szczepańska A, Korzekwa A. (2015a). Expression of pluripotency markers in the bovine uterus with adenomyosis. Reprod Biol Endocrinol. 29; 1:110.

18. Łupicka M, Bodek G, Shpigel N, Elnekave E, Korzekwa AJ. (2015b) Identification of pluripotent cells in bovine uterus: in situ and in vitro studies. Reproduction, 149; 317-327.

19. Maia L, Landim-Alvarenga FC, Mota LSLS, Golim MA, Laufer-Amorim R, De Vita B, Barberini DJ, Listoni AJ, Moraes CN, Heckler MCT, Amorim RM. (2013). Immunophenotypic, immunocytochemistry, ultrastructural and cytogenetic characterizatin of mesenchymal stem cells from equine bone marrow. Micros Res Tech; 76:618-624

20. Mambelli LI, Winter GHZ, Kerkis A, Malschutzky E, Mattos RC, Kerkis I. (2013). A novel strategy of mesenchymal stem cells delivery in the uterus of mares with endometrosis. Theriogenology; 79: 744-750.

21. Meng X, Ichin TE, Zhong J, Rogers A, Yin Z, Jackson J, Wang H, Ge W, Bogin V, Chan KW, Thébaud B, Riordan NH. (2007). Endometrial regenerative cells: A novel stem cells population. J Transl Med; 5:1-10.

22. Mensing N, Gasse H, Hambruch N, Haeger JD, Pfarrer C, Staszyk C. (2011). Isolation and characterization of multipotent mesenchymal stromal cells from the gingiva and the periodontal ligament of the horse. BMC Vet Res;7:1-13.

23. Miernik K, Karasinski J. (2012). Porcine uterus contains a population of mesenchymal stem cells. Reproduction; 143:203-209.

24. Ranera B, Ordovás L, Lyahyai J, Bernal ML, Fernades F, Remacha AR, Romero A, Vázquez FJ, Osta R, Cons C, Varona L, Zaragoza P, Martin-Burriel I, Rodellar C. (2012).

Comparative study of equine bone marrow and adipose tissuederived mesenchymal stromal. Equine Vet J; 44:33-42.

25. Rossi B, Merlo B, Colleoni S, Iacono E, Tazzari PL, Ricci F, Lazzari G, Galli C. (2014). Isolation and in Vitro characterization of bovine amniotic fluid derived stem cells at different trimesters of pregnancy. Stem Cell Rev; 10:712-724.

26. Santamaria X, Massasa EE, Feng Y, Wolff E, Taylor HS. (2011). Derivation of insulin producing cells from human endometrial stromal stem cells and use in the treatment of murine diabetes. Mol. Ther. 19, 2065-2071.

27. Stenger EO, Krishnamurti L, Galipeau J. (2015). Mesenchymal stromal cells to modulate immune reconstitution early post-hematopoietic cell transplantation. BMC Immunology, 16:1-10.

28. Souza D, Rivera L, Quevedo C, Gorino AC, Biagio S, Laufer R. (2014). Pulmonary adenocarcinoma in cattle. Revista MVZ Córdoba, 19:4358-4363.

29. Toupadakis CA, Woung A, Genetos DC, Cheung WK, Borjesson DL, Leach JK, Owens SD, Yellowley CE. (2010). Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. Am J Vet Res; 71:1237-1245.

30. Verdi J, Tan A, Shoae-Hassani A, Seifalian AM. (2014). Endometrial stem cells in regenerative medicine. J Biol Eng; 8:1-10.

31.Wollf EF, Gao XB, Yao KV, Andrews ZB, Du H, Elsworth JD, Taylor HS. (2011). Endometrial stem cell transplantation restores dopamine production in a Parkinson's disease model. J. Cell. Mol. Med. 15:747-755.

32. Xiong H, Bai C, Wu S, Gao Y, Lu T, Hu Q, Guan W, Ma Y. (2014). Biological characterization of mesenchymal stem cells from bovine umbilical cord. Anim Cells Syst; 18:56-67.

Figures

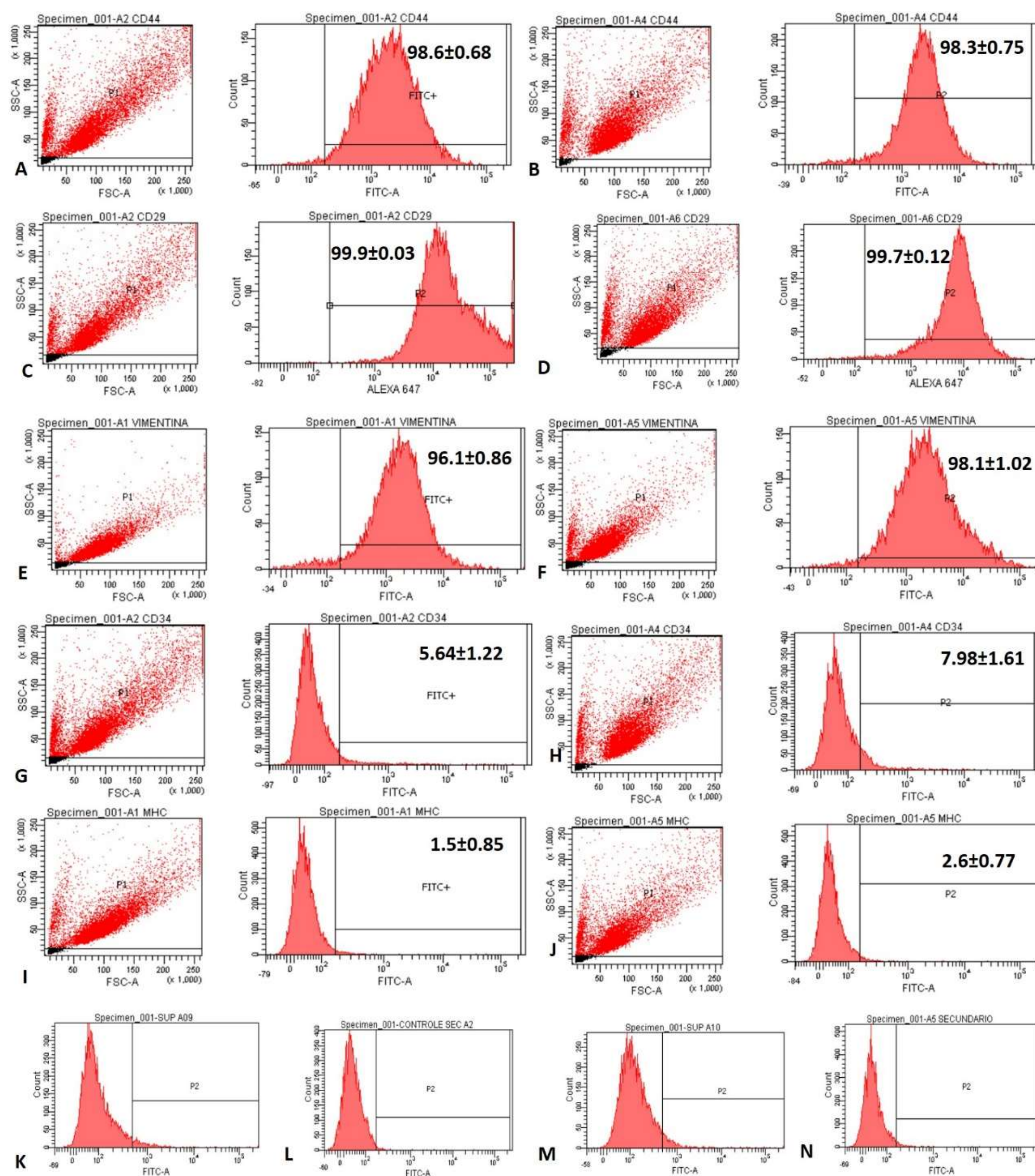


Figure 1: Immunophenotypic analysis for the markers CD44, CD29, vimentin, CD34, and MHC-II from samples of bovine eMSCs from Phase II (A, C, E, G, I) and Phase III (B, D, F, H, J). Representative histograms of marker CD44 from phase II (A) and III (B). Representative histograms of marker CD29 from phase II (C) and III (D). Representative histograms of marker vimentin from phase II (E) and III (F). Representative histograms of marker CD34 from phase II (G) and III (H). Representative histograms of marker

MCH-II from phase II (I) and III (J). Representative histograms of isotype control IgG from phase II (K) and III (M). Representative histograms of secondary control from phase II (L) and III (N). Data is presented as mean and SEM. There were no differences between groups ($p>0.05$).

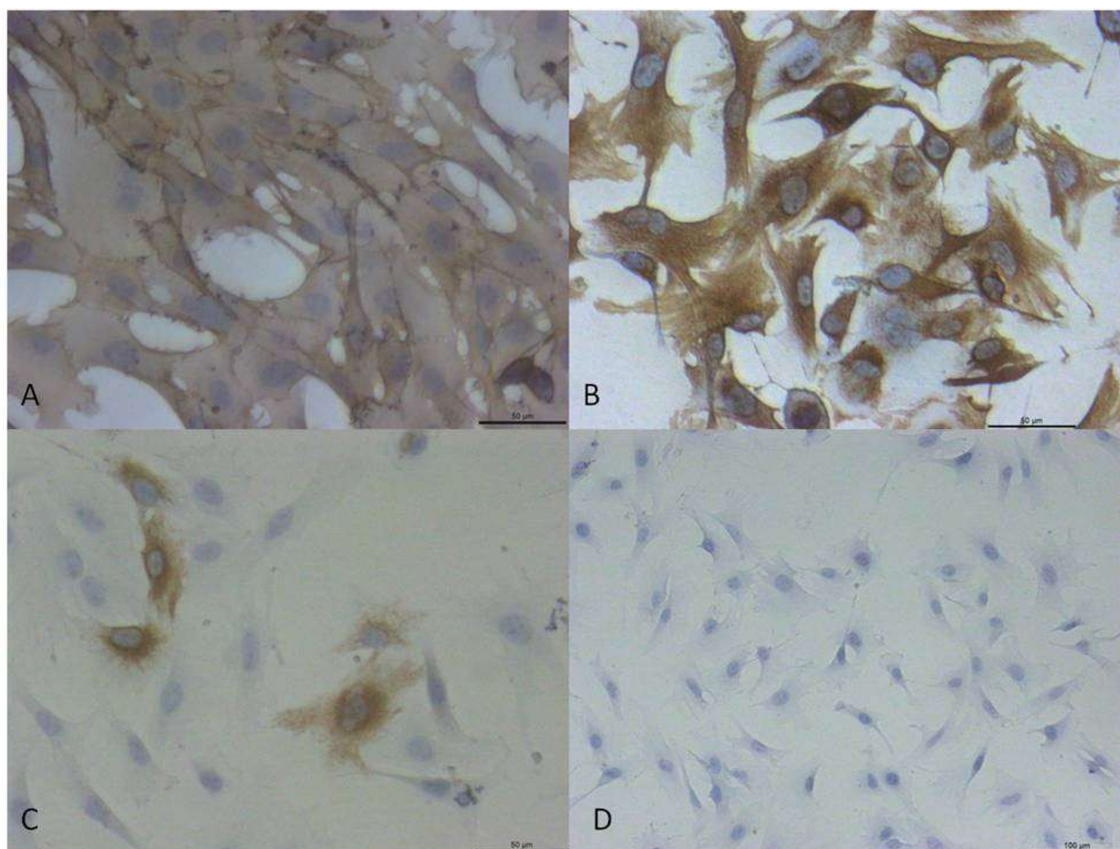


Figure 2: Immunohistochemistry of bovine eMSCs for characterization of the markers CD44 (A), vimentin (B) and cytokeratin (C). The positive staining is shown in brown. Negative control (D) by omission of the primary antibody. Nucleus stained with hematoxylin. Bar = 50 µm (A.B.C), 100 µm (D).

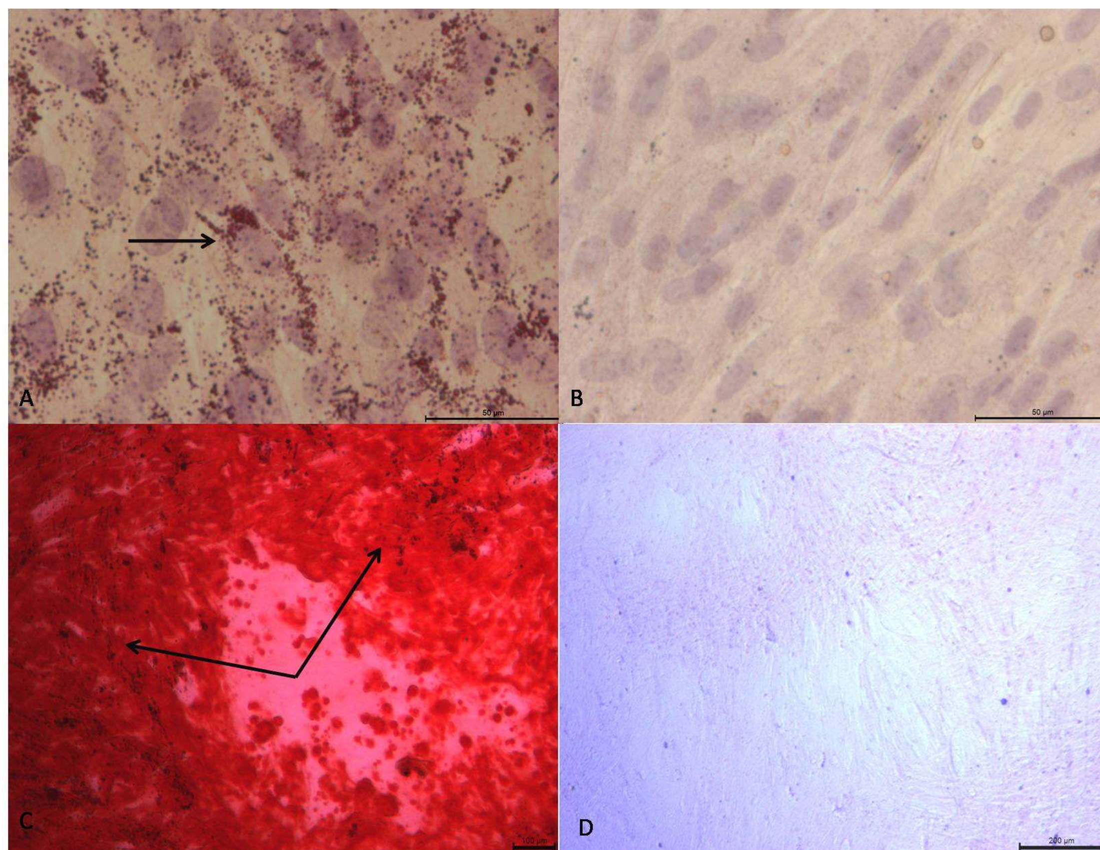


Figure 3: Differentiation assay for adipogenic and osteogenic lineages of bovine eMSCs. Adipogenic differentiation: Note in (A) the presence of intracytoplasmic lipids droplets stained with Oil red (arrow) and (B) control of differentiation. Osteogenic differentiation: Note in large calcium deposit stained with Alizarim Red (2%) (arrow) (C) and (D) control of differentiation. Bar: 100 µm (A, C) 200 µm (B, D).

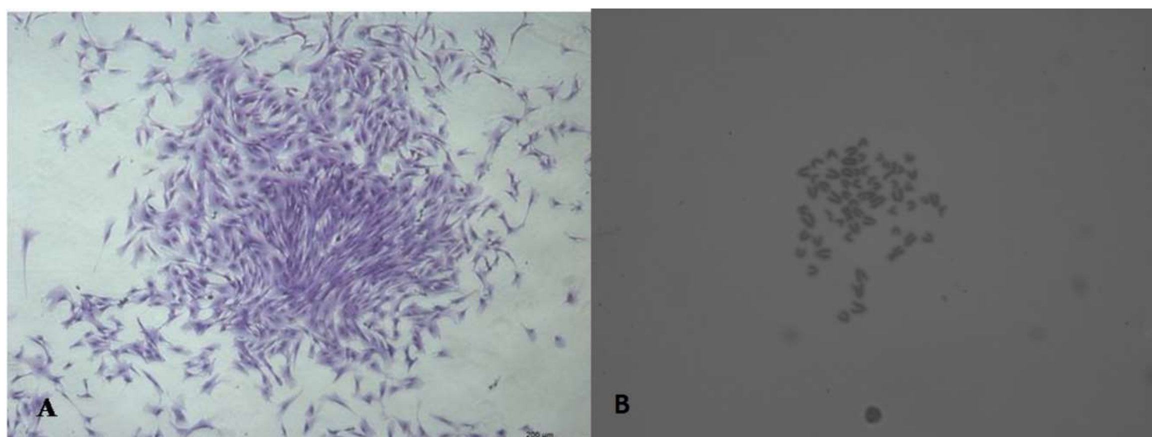


Figure 4. (A) Clonicity efficiency of bovine eMSCs. Note a formation of a colony well defined. (B) Karyotype analysis of bovine eMSCs (2n=60).

Table1: Data before and after cryopreservation analysis on flow cytometry using anexin V an propidium iodide (PI) from samples of bovine endometrial mesenchymal stem/progenitor cells from Phase II and Phase III. Data is presented as mean and SEM.

Variables	Phase II	Phase III	Phase II		Phase III	
	Before cryopreservation		After cryopreservation			
			Medium 1	Medium 2	Medium 1	Medium 2
Viability (PI- AN-)	85.08±3.88*	93.76±1.09*^{a}	81.72±2.32	78.22%±2.5	76.08%±6.48^{b}	67.18%±7.39^{b}
Necrosis (PI+AN-)	2.12±0.78	3.00±1.12	3.92%±0.79	5.68%±2.51	2.6%±0.57	1.62%±0.15
Late-apoptosis + necrosis (PI+ AN+)	4.54±1.01^{a}	1.72±0.58^{a}	11.18%±2.27^{b}	13.28%±0.99^{b}	13.02% ±3.36^{b}	21%±4.77^{b}
Initial apoptosis (PI-AN+)	3.78±0.87*	1.48±0.72*	3.14% ±1.09	2.86% ±0.67	8.26% ±3.09	10.14%±3.553

AN: anexin V; PI: propidium iodide. Different letters between the same phase and medium in the same raw shows statistical difference ($P<0.05$) before and after cryopreservation. Asterisks represents statistical difference ($P<0.05$) between phases before cryopreservation.

CAPÍTULO 2

Manuscrito publicado no periódico “Veterinary Immunology and Immunopathology”

Shotgun proteomic analysis of the secretome of bovine endometrial mesenchymal progenitor/stem cells challenged or not with bacterial lipopolysaccharide

Carolina Nogueira de Moraes[1], Leandro Maia[1], Eliandre de Oliveira[2], Camila de Paula Freitas Dell´Aqua[1], Aspinas Chapwanya[3], Fernanda da Cruz Landim-Alvarenga[1], Eunice Oba[1]

[1]Department of Animal Reproduction and Radiology, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil

[2] Proteomics Platform, Parc Cientific de Barcelona, Barcelona, Spain.

[3]Ross University School of Veterinary Medicine, Department of Clinical Sciences, Basseterre, St Kitts and Nevis

*Corresponding author:

carolnmoraes@hotmail.com

Address: Rubião Jr, s/n. Botucatu, SP/Brasil

Telephone/Fax number: 55(14) 3880-2119

Abstract

The use of the conditioned medium (CM) for diseases treatment is based on its enrichment with biomolecules with therapeutic properties and themselves have a beneficial effect. Secretome of bovine endometrial mesenchymal progenitor/stem cells (eMSCs) using a proteomics approach is until now, unknown. This work aimed to evaluate the secretome of bovine eMSCs-CM challenge or not with lipopolysaccharide (LPS). For this, eMSCs characterized were challenged (TG) or not (CG). The CM was collected 12 hours after stimulation and submitted to mass spectrometry analysis. The classification of identified proteins was done by PANTHER according to biological processes, molecular function, cellular component and protein class. 397 protein groups were identified on TG and 302 on CG. We observed positive enrichment for antibacterial response proteins, macrophage activation function, receptor-mediated endocytosis, hydrolase activity, inhibitory enzyme in TG, and for activity structural molecule and intermediate filament cytoskeleton in the CG. Our experimental model shows that eMSCs respond to LPS in the concentration used and is efficient to study immune-inflammatory response, besides of the secretion of proteins mainly related to tissue remodeling, immune response and angiogenesis which is an interesting feature for use in cell therapy.

Keywords: endometrial mesenchymal progenitor/stem cells, secretome, nanoLC-MS/MS

Introduction

In cattle, endometrial compartment is the main source of mesenchymal progenitor/stem cells (MSCs) of endometrial origin (eMSCs) with the presence of a small population of undifferentiated cells with high plasticity (Lupicka et al., 2015). Bovine eMSCs have been studied because of their biological properties including the paracrine and immunomodulatory effects, which make them promising for use in cell therapy.

Besides the use of MSCs in therapies, the conditioned medium (CM) presents role in the microenvironment regenerative and by itself is sufficient to exert a therapeutic effect in accelerating the regeneration process (Lavoie & Rosu-Myles, 2013) and tissue repair (Ashiba et al., 2015). MSCs secrete bioactive molecules (cytokines and growth factors) (Ashiba et al., 2015) released as soluble factors or through extracellular vesicles that together are responsible for the paracrine (Lavoie & Rosu -Myles, 2013) and autocrine effects related to the regenerative process, angiogenesis and modulation of immune systems (Skalnikova, 2013).

MSCs are sensitive to culture medium and protein profile may change in response to microenvironments to which they are subjected. The use of different immunological conditions evaluates the therapeutic potential of MSC-derived molecules and make the study of soluble secreted factors important for the understanding its therapeutic effect (Lavoie & Rosu-Myles, 2013). The use of an experimental *in vitro* model of inflammation using bacterial lipopolysaccharide (LPS) (Lange-Consiglio et al., 2015) makes possible to evaluate the response of bovine

eMSCs facing one stress that can bring great economic losses in cattle such as uterine diseases.

Knowledge of the composition of bovine eMSCs-CM forward to this stimulus is of great importance to the therapeutic approach and understanding of the composition for use of CM or cells in treating reproductive disease and others.

The aim of this study was to evaluate the secretome of characterized bovine eMSCs challenged or not with bacterial LPS by proteomic analysis (nanoLC-MS/MS) using a shotgun strategy. To the best of our knowledge, this is the first report describing the secretome of bovine eMSCs - CM using proteomic analysis.

Material and methods

The study was performed according to the ethical guidelines and it was approved by the institution's Animal Care and Experimentation Ethics Committee (Protocol Number 152/2014).

Isolation and culture of bovine eMSCs

Samples from bovine endometrial tissue (n=3) on Phase II of estral cycle (Ireland et al., 1980; Chapwanya et al., 2013) were properly isolated according to methodology from Fortier et al. (1988) with modifications. For this, samples were submitted to a first digestion with 0.3% trypsin (Sigma ®, USA) in 10 ml of HBSS (Thermo Fisher Scientific®, USA) for 3 hours at 22°C under agitation and filtered with 40 uM filter (Becton Dickinson® and Company, USA). A second digestion was done with 50 mg of trypsin, 50 mg of collagenase, 100 mg of bovine serum albumin and 10 mg DNase I (Sigma ®, USA) in 100 mL of HBSS (Thermo Fisher Scientific®, USA) for 1.5 hours at 37°C. After new filtration, the material was washed with HBSS

medium plus 10% fetal bovine serum (FBS) (Thermo Fisher Scientific®, USA) and centrifuged (Hermle centrifuged Z200A, GER) twice at 100 x g for 10 minutes and then samples were plated and cultured with medium composed by DMEM high glucose/F12 (1:1), 20% FBS, penicillin (100IU/ml), streptomycin (100µg/ml), amphotericin B (3µg/mL) (Thermo Fisher Scientific®, USA) and amikacin (11µg/mL) (Teuto®, BRA) at 37.5°C in humid atmosphere containing 95% air and 5% CO_2. The medium was exchanged within 18 hours and then every 2-3 days until reach confluence and realization of the subcultures until 3th passage in which the assays were done.

Immunophenotypic characterization

The immunophenotypic characterization (n=2) was performed by flow cytometry at a LSR Fortessa (BD®, BR) equipment using the antibodies mouse anti–CD29: Alexa fluor 647 (TS2/16, BioLegend®, USA), mouse anti-bovine CD44:FITC (IL-A118, AbD Serotec®, UK), mouse anti-horse MHC–II:FITC (CVS20, AbD Serotec®, UK), rabbit anti CD-34:FITC (polyclonal, Biorbyt®, USA) and mouse anti-vimentin (v9, AbD Serotec®, UK) with the use of the secondary goat-anti mouse (abcam®, USA) for this last one. All antibodies used have proved reactivity for bovine. During the analysis were accounted for 10,000 events and were considered positive markers with expression greater than 2%. Data from immunophenotypic characterization is presented as mean and standard error of the mean.

Immunocytochemistry characterization

The immunocytochemistry characterization was done (n=3) according methodology described by Maia et al. (2013) for the antibodies vimentin (1: 200, V9,

AbD Serotec®, UK), pan-cytokeratin (1: 100, C11, abcam®, USA) and CD44 (1: 100, BAG40A, VMRD®, USA).

Assays for differentiation

It was done the assay for differentiation on the osteogenic (n=3) and adipogenic (n=3) lineage. After confluence, the differentiation medium for adipogenic or osteogenic was added to the subcultures in triplicate, according to manufacturer's recommendations (StemPro, Thermo Fisher Scientific®, USA) with modifications by the supplementation of 5% rabbit serum (Maia et al., 2013) or 20% FBS, respectively.

The confirmation of the osteogenic differentiation was done on the 14th day demonstrated by the presence and deposition of calcium matrix and of the adipogenic differentiation was done on 8th day demonstrated by the presence of intracytoplasmic fat droplets at the histological staining method with Alizarin red pH 4.2 (Sigma®, USA) and Oil red 0.5% (Sigma®, USA), respectively.

Challenge of bovine eMSCs with LPS

For the evaluation of the protein profile on secretome, bovine eMSCs were plated on 24 wells (2cm^2) at a density of 1000 cells/cm^2 and cultured with complete maintenance medium composed by DMEM high glucose/F12 (1:1), 20% FBS, penicillin (100IU/mL), streptomycin (100μg/mL), amphotericin B (3μg/mL) (Thermo Fisher Scientific®, USA) and amikacin (11μg/mL) (Teuto®, BRA). After approximately 60-70% confluency cells were grown in maintenance medium without FBS for 24 hours.

After 24 hours, cells were either treated with control medium (maintenance medium without FBS) (n = 3, control group- CG) or were challenged with LPS (n = 3,

treated group- TG) and therefore cultured with medium composed of DMEM high glucose (Invitrogen Gibco ®, USA) plus 1µg/mL LPS (*E. coli* serotype 0111: B4, Sigma).

After 12 hours of cultivation, the conditioned medium was collected, filtered through 22 µm filter and subjected to centrifugation at 2000 x g for 5 min to remove cellular debris. After centrifugation, the conditioned medium was put in cryotubes and stored at -86°C until completion of secretome analysis.

Secretome analysis by mass spectrometry coupled to liquid chromatography (nanoLC- MS/MS)

For proteomics analysis of CM it was used three biological replicates in both groups (treated vs. control) as pool. For digestion, the samples were initially denatured in 8 M urea solution (Sigma 51459), followed by reduction with 50 mM dithiothreitol (32°C/60 minutes, Sigma 9779), alkylation with Iodocetamida 150 mM (25°C/30 minutes in the dark) and digestion with trypsin Sequence grade (35ºC, 16 hours, Promega V511A). After digestion it was performed clean-up with C18 reversed phase and Strong Cationic-Exchange columns (C18, SCX, PolyLC). The samples (two technical replicates) were analyzed in a nanoAcquity liquid chromatographer (Waters) coupled to a LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer.

Data analysis

Thermo Proteome Discover (v.1.4.1.14) was used to search with SequestHT search engine against Mammalia-SwissProt+Bos taurus-TREMBL protein database (v. april 2016). The search parameters used were: Enzyme: Trypsin; Missed Cleavage: 2; Precursor and Fragment Mass Tolerances: 10 ppm and 0.6 Da, respectively;

Variable and Static: Oxidation methionine and Carbamidomethyl cysteine, respectively.

Gene Ontology protein classification analysis according biological process (BP), molecular function (MF), cellular component (CC) and protein class (PC) was performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (http://pantherdb.org/).

Results and Discussion

Bovine eMSCs were isolated from uterus on Phase II of estral cycle, cultured, characterized by immunophenotyping, immunocytochemistry and for potential of differentiation and submitted or not to the challenge with LPS for secretome analysis using a proteomic approach.

Bovine eMSCs showed adherence to plastic within six hours of culture and fibroblastoid morfology at all passages (Figure 1). Immunophenotypic evaluation by FC reveled high expression for the following markers: vimentin (94.35 ± 2,19), CD29 (99.85± 0.07) and CD44 (96.9 ± 2,40). On the other hand, low expression was observed for CD34 (4.25± 1,06) and OCT-4 markers (5.45± 1,91). Absence of expression was detected for MHC-II marker (1.05± 0,78) (Figure 2), which corroborates with other studies done in bovine MSCs (Xiong et al., 2014). We carried out staining methods to evaluate osteogenic and adipogenic potential of the eMSCs. Positive staining for osteogenic and adipogenic lineages was confirmed, which is in accordance with other study using the same stem cell source (Lupicka et al., 2015). Moreover, in qualitative analysis of immunocytochemistry, other authors also found a positive staining for the markers CD44 in bovine endometrial putative mesenchymal cells (Cabezas et al., 2014) and for vimentin in human eMSCs (Kato 2012). Some

epithelial cells were positively marked for pan cytokeratin, which showed no negative influence on cell culture.

A proteomic analysis was performed in order to get some qualitative information and better knowledge in terms of the protein content of the secretome of eMSCs. A shotgun approach was carried out and the LC-MS/MS results evidenced a total of 397 proteins groups on TG and 302 on CG, being 242 commons between groups, as can be observed on Figure 3. A greater number of proteins related to immune system and tissue remodeling was observed in the TG, besides of the presence of proteins related to angiogenic, antioxidant and protective activity (Table 1a).

Our *in vitro* experimental model showed that the cells respond positively and by a protective way to the exposure to LPS in the concentration used. It can be inferred, for instance, from the presence of heat shock protein, which is a protein that protect cells or tissues from stress (Frier & Locke, 2007) by stabilization and repair of proteins (Fan et al., 2012). This response is characterized by a defense mechanism via secretion of important proteins related to tissue restoration, and for the protection of tissue injury, supporting the assertion that CM alone or together with MSCs can be a great efficient alternative to assist in repair of lesions (Ashiba et al., 2015).

MSCs are sensitive to culture medium and may change their proteomic profile in response to the microenvironments in which they are subjected (Lavoie &-Rosu Myles, 2013). This was observed by our group as the samples challenged with LPS responded differently regarding the protein expression, as mentioned above. It is noteworthy that in the CM of CG were also found proteins with activities related to immune response and tissue remodeling, although in lower number on TG (Table 1). The presence of such proteins in the CM of CG, even without stimulation, is quite interesting and promising to demonstrate the great potential and possibility of using

this medium for therapeutic use. In humans, the CM of MSCs from dental pulp was efficient in the experimental treatment of multiple sclerosis and even showed similar effects of treatment with their own MSCs (Shimojima, et al., 2016). An interesting protein related to antioxidant activity (peroxiredoxin-6, Table 1C) was found at TG, showing the presence of this important function. The antioxidant capacity of CM was evidenced by Duarte et al. (2016) on the restoration and reduction of damage of retinal functions at a diabetes animal model.

Recently, Lange-Consiglio et al. (2015) showed in an experimental equine uterine inflammation model that horse amniotic MSCs-CM was able to significantly reduce expression of MMP-1 and MMP-13 and had a role in the modulation of inflammatory genes. In the present work, anti-inflammatory protein (granulins, Table 1) was also found at TG, which confirm such property. In addition, in vitro use of the CM in conjunction with endometrial cells resulted in improvement of the proliferation rate, showing the relevance of soluble factors produced by MSCs and its potential to increase cell replacement (Corradeti et al., 2014). Such studies support using CM as a great alternative for the treatment of uterine diseases related to low fertility and pregnancy rates due to restoration of damaged tissue.

To better characterize the CM proteome, we performed some classification on the identified proteins using Gene Ontology tools (http://geneontology.org/). The Panther GO analysis evidenced positive enrichment (PE) for BP in macrophage activation function (GO: 0042116, fold enrichment, FE = 7.87) and receptor-mediated endocytosis (GO: 0006898, FE = 5.23) in TG. Regarding FM, it was seen PE for hydrolase activity (GO: 0016787; FE = 1.87) and inhibitory enzyme (GO: 0004857; FE = 5.15) in the TG, and activity structural molecule (GO: 0005198; FE = 2.73) in the CG. For CC, enrichments were verified in both groups to extracellular matrix (GO:

0031012) and extracellular region (GO: 0005576), as well as intermediate filament cytoskeleton (GO: 0045111) in the CG. Interestingly, with respect to PC we evidenced PE in antibacterial response proteins (PC00051; FE = 5.87) in the TG.

The secretome of characterized bovine eMSCs challenged or not with LPS revealed that cells secrete different proteins both in terms of numbers and also function. Our results, especially based on protein enrichment to macrophage activation and identification of large number of proteins related to immune response on TG, allow us to infer that our *in vitro* model of stimulation with bacterial LPS of bovine eMSCs is effective to study immune and inflammatory response. Additionally, we believe that eMSCs and its CM are quite promising for therapeutic applications, since we observed proteins with functions related to immune response, antimicrobial, anti-inflammatory and tissue remodeling even in unstimulated cells secretome.

Acknowledgements

To Proteomics Platform of the Barcelona Science Park. To PRB2, ProteoRed-ISCIII, the Spanish Proteomics network. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo [Proc.2014/20447-2, Proc. 2015/18964-1, Proc.2015/01057-1).

References

Ashiba, K., Terunuma, A., Terunima, H., Takane, T., Deng, X., Yamashita, Y., Watanabe, K., 2015. Immortalized mesenchymal stem cells producing conditioned medium in a large scale for -t, herapeutic useage. Inflamm Regen. 35, 57-60. http://doi.org/10.2492/inflammregen.35.057.

Cabezas, J., Lara, E., Pacha, P., Rojas, D., Veraguas, D., Saravia, F., Rodríguez-Alvarez, L., Castro, F.O., 2014. The endometium of cycling cows contains populations of putative mesenchymal progenitor cells. Reprod Dom Anim. 49, 550-559. doi: 10.1111/rda.12309.

Corradetti, B., Correani, A., Romaldini, A., Marini, M.G., Bizarro, D., Perrini, C., Cremonesi, F., Lange-Consiglio, A., 2014. Amniotic membrane-derived mesenchymal cells and their conditioned media: Potential candidates for uterine regenerative therapy in the horse. Plos One. 9, 1-9. doi: 10.1371/journal.pone.0111324.

Donofrio, G., Franceschi, V., Capocefalo, A., Cavirani, S., Sheldon, I.M., 2008. Bovine endometrial stromal cells display osteogenic properties. Reprod Biol Endocrinol. 6, 1-9. doi: 10.1186/1477-7827-6-65.

Duarte, D.A., Papadimitriou, A., Gilbert, R.E., Thai, K., Zhang, Y., Rosales, M.A.B., Faria, J.B.L., Faria, J.M.L., 2016. Conditioned medium from early- outgrowth bone marrow cells is retinal protective in experimental model of diabetes. PLoS One. 11, 1-15. doi: 10.1371/journal.pone.0147978.

Fan, G.C., 2012. Role of heat shock proteins in stem cell behavior. Prog Mol Biol Transl Sci. 11, 305-322. doi: 10.1016/B978-0-12-398459-3.00014-9.

Frier, B.C., Locke, M., 2007. Heat stress inhibits skeletal muscle hypertrophy. Cell Stress Chapereones, 12, 132-141. doi: 10.1379/CSC-233R.1.

Lange-Consiglio, A., Perrini, C., Esposti, P., Derigibus, M.C., Camussi, G., Pascucci, L., Marini, M.G., Corradetti, B., Bizarro, D., Cremonesi, F., 2015. Effects of microvesicles secreted from equine amniotic-derived progenitor cells on in vitro lipopolysaccharide-treated tendon and endometrial cells. Reprod. Fertil. Dev., 28, 244-245. http://dx.doi.org/1 0.1 071 /RDv28n2Ab226.

Lavoie, J.R., Rosu-Myles, M., 2013. Uncovering the secretes of mesenchymal stem cells. Biochimie, 95, 2212-2221. doi: 10.1016/j.biochi.2013.06.017.

Lupicka, M., Bodek, G., Shpigel, N., Elnekave, E., Korzekwa, A.J., 2015. Identification of pluripotent cells in bovine uterus: in situ and in vitro studies. Reproduction, 149, 317-327. doi: 10.1530/REP-14-0348.

Shimojima, C., Takeuchi, H., Jin, S., Parajuli, B., Hattori, H., Suzumura, A., Hibi, H., Ueda, M., Yamamoto, A., 2016. Conditioned medium from the stem cells of human exfoliated deciduous teeth ameliorates experimental autoimune encephalomyelitis. J Immunol., 15, 4164-4171. doi: 10.4049/jimmunol.1501457

Skalnikova, H.K.,2013. Proteomic techniques for characterization of mesenchymal stem cell secretome. Biochimie, 95, 2196-211. doi:10.1016/j.biochi.2013.07.015.

Xiong, H., Bai, C., Wu, S., Gao, Y., Lu, T., Hu, Q., Guan, W., Ma, Y., 2014. Biological characterization of mesenchymal stem cells from bovine umbilical cord. Anim Cells Syst., 18, 56-67. http://dx.doi.org/10.1080/19768354.2014.880370.

Kato, K. 2012. Stem cells in human normal endometrium and endometrial cancer cells: Characterization of side population cells. Kaohsiung J Med Sci, 28,63-71. doi:10.1016/j.kjms.2011.06.028

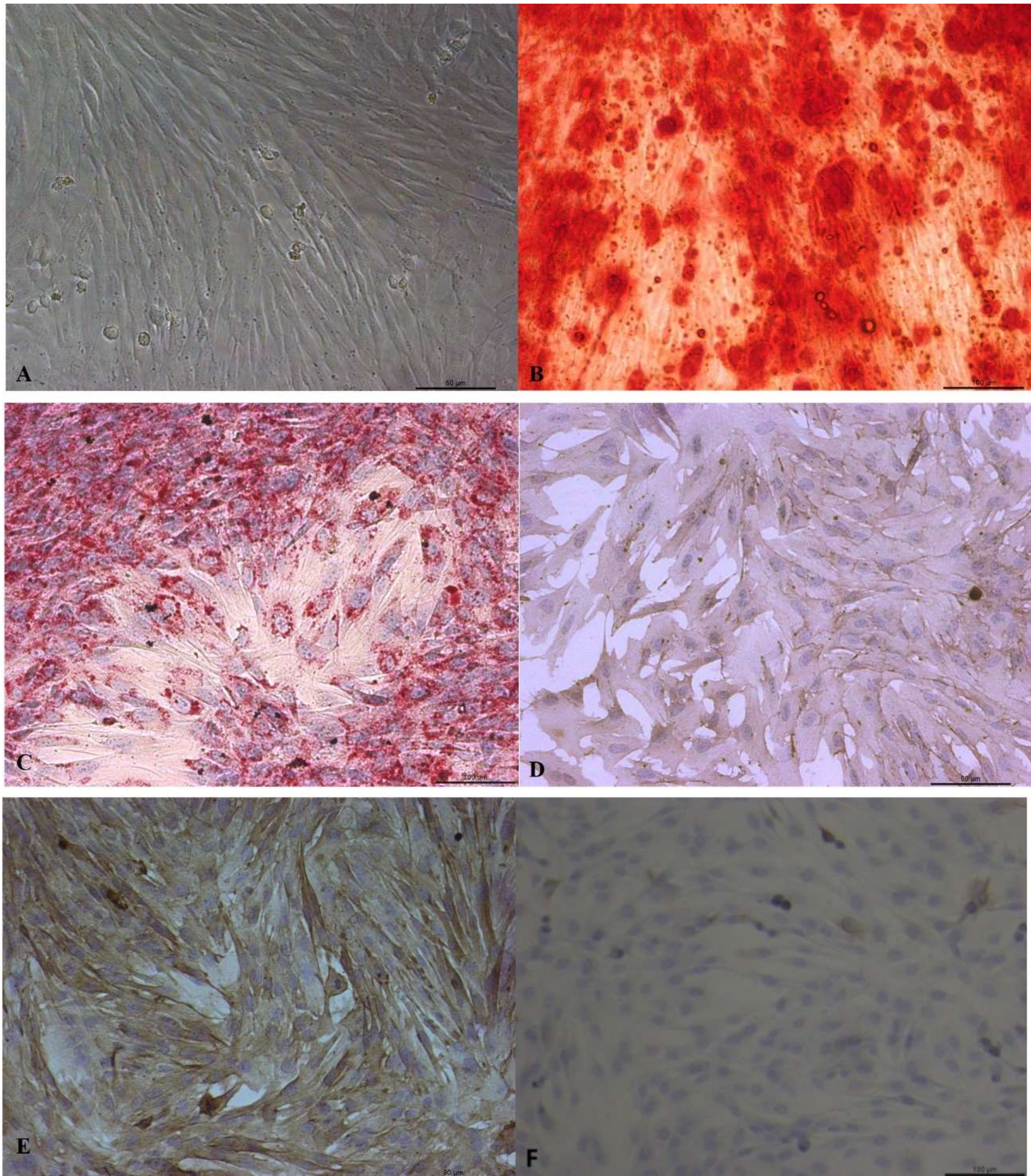


Figure 1: Characterization of bovine eMSCs. Bovine eMSCs on first passage presenting fiblobastoid morfology and adherence to plastic (A). Differentiation assay into osteogenic lineage of bovine eMSCs in third passage (B). Note calcium deposit stained with Alizarim (arrow). Differentiation assay into adipogenic lineage of bovine eMSCs in third passage (C). Note the intracytoplasmic lipids droplets stained with Oil red (arrow). Immunohistochemistry of bovine eMSCs for CD44 (D), vimentin (E) e

pancytokeratin (F). Positive staining is observed in brown. Nucleus stained with hematoxylin. Size: 100 µm (A, D, F), 200 µm (B, C, E).

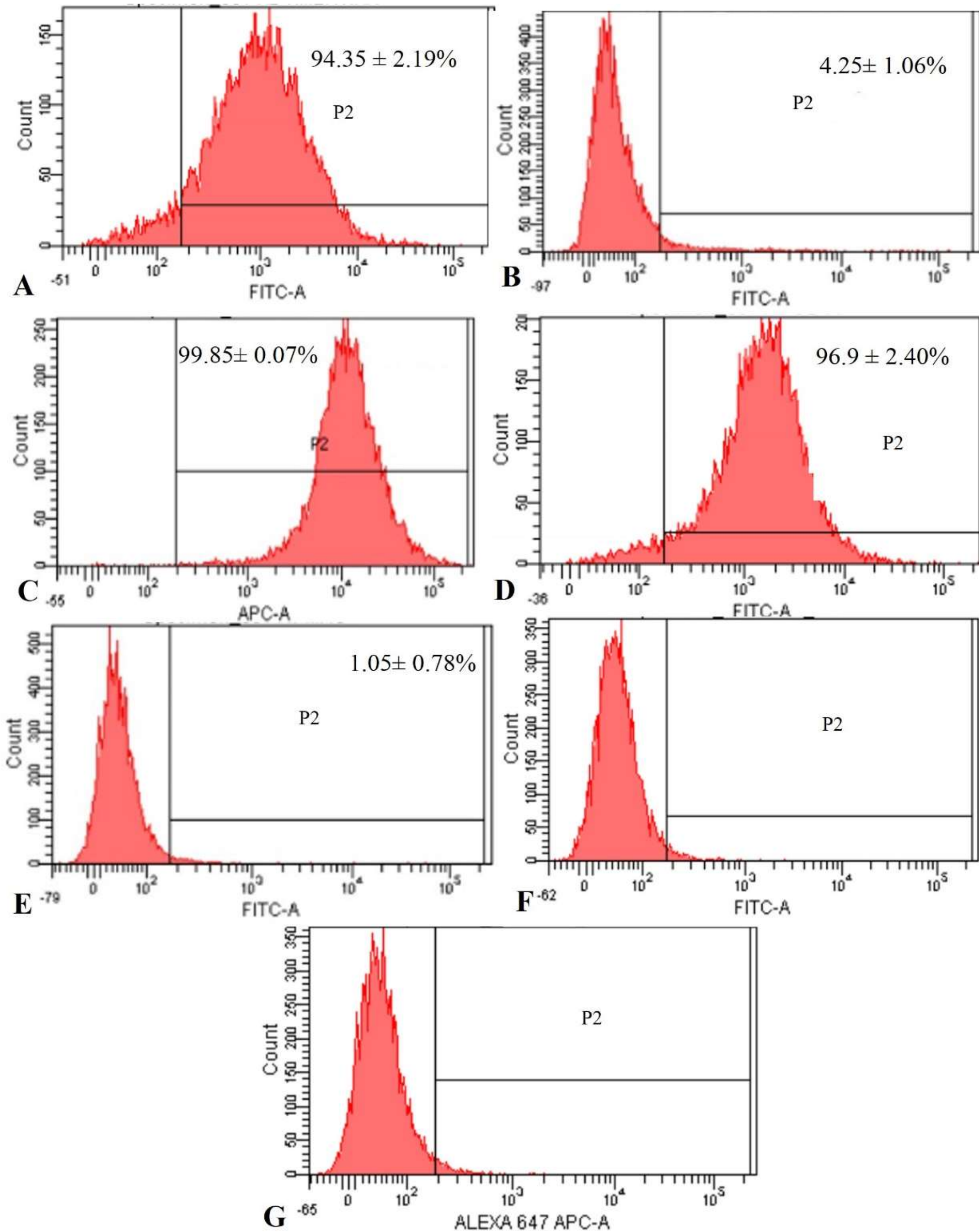


Figure 2: Representative histogram for the markers analyzed by flow cytometry on bovine eMSCs: vimentin (A), CD29 (B), CD44 (C), CD34 (D), OCT-4 (E) and MHC-II (F).

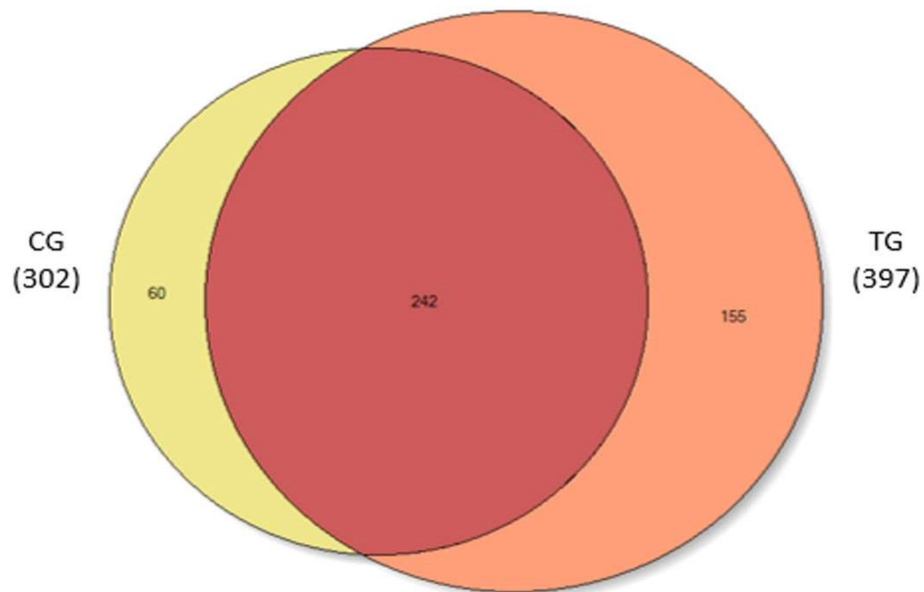


Figure 3: Venn Diagram of proteins identified with at least one peptide sequence (False discovery rate≤1%) on studied groups. TG: treated group; CG: control group.

Table 1: Protein identified in the secretome of conditioned medium of bovine eMSCs treated or not with LPS.

Biological Functions	Swiss-Prot accession	Protein name	Gene name	Group
Immune response	Q1JPB0	Leukocyte elastase inhibitor	SERPINB1	Control
	A2I7M9	Serpin A3-2	SERPINA3-2	Control
	A5D7R6	ITIH2 protein	ITIH2	Control
	F1MVS9	Uncharacterized protein	MASP1	Control
	F1MNV5	Kininogen-1	KNG1	Control
	Q3SYR0	Thyroxine-binding globulin	SERPINA7	Control
	A0A0A0MP92	Serpin A3-7	SERPINA3-7	Control
	G3N3P6	Cystatin	N/A	Treated
	F1MI18	Uncharacterized protein	N/A	Treated
	P05089	Arginase-1	ARG1	Treated
	Q2KIF2	Leucine-rich alpha-2-glycoprotein 1	LRG1	Treated
	A7YWB6	Transforming growth factor-beta-induced protein	TGFBI	Treated
	A5PJE3	ig-h3	FGA	Treated
	E1B726	Fibrinogen alpha chain	PLG	Treated
		Plasminogen		
Antimicrobian / antifungal activity	P03973	Antileukoproteinase	SLPI	Control
	P59665	Neutrophil defensin 1	DEFA1	Control
	P06702	Protein S100-A9	S100A9	Control
	P81644	Apolipoprotein A-II	APOA2	Treated
Anti-inflammatory activity	A5A6M2	Annexin A1	ANXA1	Control
	P2879	Granulins	GRN	Treated

Biological Functions	Swiss-Prot accession	Protein name	Gene name	Group
Tissue remodeling	P03973	Antileukoproteinase	SLPI	Control
	A5A6M2	Annexin A1	ANXA1	Control

	E2DI12	Syndecan-1 (Fragment)	SDC1	Control
	P28799	Granulins	GRN	Treated
	P50757	72 kDa type IV collagenase	MMP2	Treated
	E1B726	Plasminogen	PLG	Treated
	F1N2Y2	Uncharacterized protein	COL5A2	Treated
	P05089	Arginase-1	ARG1	Treated
	A5PJE3	Fibrinogen alpha chain	FGA	Treated
Angiogenic activity	P80929	Angiogenin-2	ANG2	Treated
	Q2KIF2	Leucine-rich alpha-2-glycoprotein 1	LRG1	Treated
	P50757	72 kDa type IV collagenase	MMP2	Treated
Protective activity	E9RHW1	Heat shock 27kDa protein 1	HSPB1	Treated
Antioxidant	P86215	Peroxiredoxin-6	PRDX6	Treated

CAPÍTULO 3

Manuscrito nas normas, a ser submetido no periódico “Cytokine”

Link para as normas: https://www.elsevier.com/journals/cytokine/1043-4666/guide-for-authors

TITLE PAGE

TITLE: Evaluation of the immunomodulatory properties of bovine endometrial mesenchymal stem cells

Carolina Nogueira de Moraes[a*], Leandro Maia[a], Paula Fernanda de Lima[b], José Buratini Junior[b], Felipe Rydyguer de Ruediger[a], Fernanda da Cruz Landim[a], Eunice Oba[a]

[a] Department of Animal Reproduction and Veterinary Radiology, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil

[b] Department of Pharmacology, Biosciences Institute, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil

*Corresponding author:

carolnmoraes@hotmail.com

Address: Rubião Jr, s/n. Botucatu, SP/Brasil

Telephone/Fax number: 55(14) 3880-2119

Abstract

Bovine endometrial mesenchymal stem cells (eMSCs) is a new studied source of progenitor cells that could be used at therapeutic approaches mainly due its immunomodulatory effect. The present work aimed to evaluate gene expression, cytokines and prostaglandin E_2 (PGE_2) production after challenge with bacterial lipopolysaccharide (LPS). For this, bovine eMSCs already characterized were cultured until third passage and after reach confluence; were divided into studied groups and cultured with control medium (C) or with 1µg/mL LPS (T) diluted in medium. Conditioned medium (CM) was collected after 2, 6, 12 and 24 hours of exposition, filtered and centrifuged for cytokine analysis (INF-α, INF-γ, IL-13, IL-1α, IL-F5, IL-21, MIP-1β and TNF-α). The CM collected at 2 and 24 hours was also used for PGE_2 analysis. In addition, cells on third passage were analyzed for vimentin CD44, IL-1α and prostaglandin-endoperoxide synthase 2 (PTGS2/COX-2) expression. Results showed that eMSCs produce all analyzed cytokines, with higher ($P<0.05$) production of TNF-α on T group. Additionally, PGE_2 analysis revealed higher ($P<0.05$) production after 24 hours of stimulus and cells also expressed all studied genes. Based on our results, we can infer that this source of MSCs are able to respond to a challenge with LPS, producing cytokines related to immunomodulatory effect and also PGE_2, corroborating for tissue repair in case of damage that can be caused during inflammatory processes such as endometritis.

Keywords: cow, cytokine, immunomodulation, LPS, PGE_2

Abbreviation:

CM: conditioned medium

eMSCs: endometrial mesenchymal stem cells

FBS: fetal bovine serum

INF: interferon

IL: interleukin

LPS: lipopolysaccharide

MSCs: Mesenchymal stem cells

TNF- α: tumoral necrosis factor-α

1. Introduction

Mesenchymal stem cells (MSCs) have immunomodulatory effects demonstrated by interaction between cells, secretion of soluble factors such as growth factors, cytokines and chemokines or by interaction of both [1]. The effect of MSCs depends upon the microenvironment at damaged tissues and on engagement of toll-like receptors expressed by MSCs, determining their pro or anti-inflammatory action [2] and also inflammation repair or persistence [3].

The secretion of cytokines, immunosuppressive and growth factor are part of a complex network that helps amplify response against a stimulus [4]. MSCs can release these molecules crucial for immunoregulation (such as prostaglandin E_2 (PGE_2), interleukin 10 (IL-10), transforming growth factor-β) and tissue repair spontaneously or after induction by cytokine (mainly interferon γ (INF- γ), tumoral necrosis factor-α (TNF-α), IL-1α) or lipopolysaccharide (LPS) [3]. In addition, the conditioned medium (CM), have important proteins related to immune-inflammatory and angiogenic properties beside proteins that participates at tissue remodeling [5], which makes its therapeutic use promising.

Cattle is a good model to study uterine infections effects on endocrine and immune functions, once diseases are well characterized, tissue is easily collected and there is

similarity of uterine bacteria among mammals [6]. Uterine infections have a negative effect on reproduction once affects ovarian activity and may contribute to uterine diseases and fertility problems [7]. The *Escherichia coli* bacteria is the most important pathogen that leads to this affection, responsible for endotoxin (LPS) production [8] that activates innate immune response [6]. At this context, the understanding of how cells respond to an infection or damage is of great importance to preventing and treating endometrial diseases [9].

The knowledge of the soluble factors produced by MSCs on CM upon stimulus are of great value for knowledge of MSCs biology and therapeutic application. This study aimed to evaluate cytokines and prostaglandin E_2 production by endometrial MSCs (eMSCs) under challenge or not with LPS. Additionally evaluate the expression of genes of characterization and inflammation on eMSCs. This work begins from the hypothesis that spontaneous production of cytokines in norm conditions of culture is low, thus requiring stimulating conditions, as a stimulus by LPS for eMSCs performing their immunomodulatory activity.

2. Material and Methods

This study was approved and performed according to ethical guidelines of the Institution’s Animal Care and Experimentation Ethics Committee (Protocol Number 152/2014).

2.1 Endometrial mesenchymal stem cells isolation, characterization and culture

The isolation, culture and characterization of eMSCs were done, as previously described [10]. After isolation, eMSCs were cultured until third passage, for cytokine and

PGE_2 analysis on CM, and quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) on cells. A timeline is presented on Figure 1.

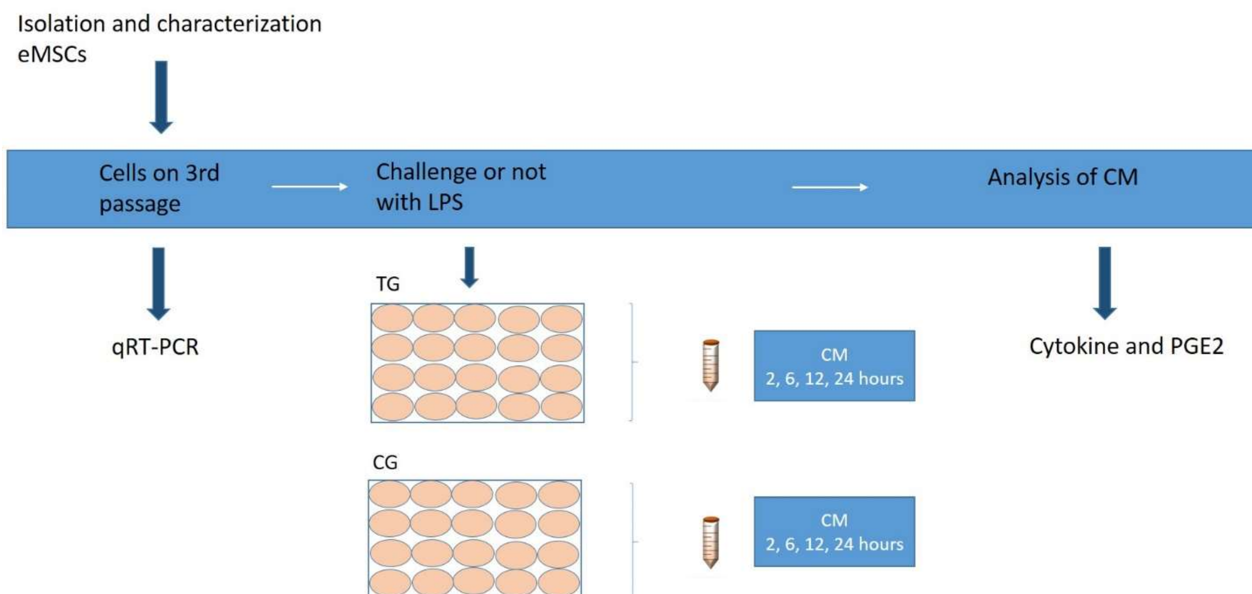


Figure 1: Timeline. Endometrial mesenchymal stem cells were collected and characterized. Cells on third passage were submitted to quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) and to stimulus (TG) or not (CG) with bacterial lipopolysaccharide (LPS). Conditioned medium (CM) was collected after 2, 6, 12 and 24 for cytokine and PGE_2 analysis.

2.2 Quantitative reverse transcriptase–polymerase chain reaction

For qRT-PCR, $1x10^6$ eMSCs (n=12) from luteal phase [12, 13] were cultured on six wells plates, until confluence when were then trypsinized, resuspended at 300 µL of lysis solution (Qiagen, USA) and stored in 1.5 ml sterile tubes at -80°C.

Total RNA extraction from cells was performed using Rneasy® Mini Kit (Qiagen, USA), according to manufacturer's recommendations. After purification, RNA samples were eluted in 40 µL RNAse-free water. Total RNA concentration was measured at a spectrophotometer using NanoDrop ND® 1000 (Thermo Scientific®, USA), and stored at -80°C until analysis.

After quantification, total RNA was incubated with DNAse I (1 U/µg; Invitrogen®, BRA) and then reverse transcribed with Oligo-dT primers and Omniscript RT Kit (Qiagen®, USA) for obtain complementary DNA (cDNA). The reagents were incubated at 37 ºC for 60 min and then at 93 ºC for 3 min for enzyme inactivation. Relative real time RT-PCR analysis was performed in 96-well plates with an ABI 7500 thermocycler using Power Sybr Green PCR Master Mix (Applied Biosystems, BRA), cDNA, and primers.

Cycling conditions were: 95°C/10 minutes (1 cycle), denaturing at 95°C/10 seconds, and annealing for 1 minute (40 cycles). Housekeeping genes cyclophilin A and glyceraldehyde-3-phosphate dehydrogenase (GADPH) as internal positive control for the qRT-PCR. The target gene expression was thus normalized to expression of the housekeeping gene GAPDH. Vimentin, CD44, IL-1α and prostaglandin-endoperoxide synthase 2 (PTGS2/COX-2) mRNA were evaluated on cells and sequence primers and product size are demonstrated on Table 1.

Samples were run in duplicate, and relative expression values for each gene were calculated using ΔΔCt method with efficiency correction and one control sample as calibrator [14].

Table 1: Sequence of primers, annelling temperature and amplification product sizes of genes used to evaluate bovine eMSCs.

Gene	Primer sequence	Anneling temperature	Product size (bp)	Reference
Vimentin	F: GATGTTTCCAAGCCTGACCTC R: GGCGTTCCAGAGACTCGTTAG	60	253	[15]
CD44	F: GACCCTCAATCTTCCTCTTCAC R: TTTCCTACCAGGTGCCAATC	60	94	NM_174013.3
PTGS2	F: AAGCCTAGCACTTTCGGTGGAGAA R: TCCAGAGTGGGAAGAGCTTGCATT	60	168	[16]
IL-1α	F: GATGCCTGAGACACCCAA R: GAAAGTCAGTGATCGAGGG	60	173	[17]
GADPH	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT	54	119	[18]

F: forward, R: reverse

2.3 Stimulation of eMSCs with LPS

For cytokine assay and PGE_2 analysis, eMSCs (1000 cells/ cm^2/24 wells plates) were cultured in medium composed of DMEM high glucose/F12 (1:2), 20% fetal bovine serum (FBS), 100IU/mL penicillin, 100µg/mL streptomycin, 3µg/mL amphotericin B; Thermo Fisher Scientific®, (USA), 11µg/mL amikacin Teuto®, (BRA), until 60% confluence. After it, cells were maintained with FBS free medium for 24 hours.

After 24 hours, control (CG; n=10, medium FBS free) and LPS-stimulated cells (TG; n=10, 1µg/mL [11] (E. coli, serotype 0111:B4, Sigma) were cultured. The medium was collected after 2, 6, 12 and 24 hours of stimulus, filtered through 22 µm filter and centrifuged at 2000 x g for 5 minutes. It was stored at -80°C until analysis.

2.4 Quantification of Cytokines

Samples (n=10) of CM collected after 2, 6, 12 and 24 hours of exposition or not to LPS were used to evaluate cytokines production. Samples were analyzed by Quantibody® Bovine Cytokine Array 1 kit (QAB-CYT-1, Raybiotech®, EUA), valid for bovine species, according to manufacturer's specifications. This array evaluated INF-α, INF-γ, IL-13, IL-1α, IL-F5, IL-21, MIP-1β and TNF-α concentration.

The slides were scanned by using a microarray scanner (Innopsys, model InnoScan 710) and images were analyzed using Mapix 7.0 software (Innopsys).

Each sample was evaluated in quadruplicate, and their data were normalized according to intra-slides positive controls. Results were achieved by eliminating the background and are presented as median with interquartile rage. Sample concentration (pg/mL) was calculated based on linear regression of standard curves.

2.5 Prostaglandin E_2 concentration

The concentration of PGE_2 was evaluated on CM cell-free previously collect 2 and 24 hours after stimulation or not with LPS. Analysis were done using PGE_2 ELISA kit (Enzo®, USA), according to the manufacturer's recommendations. Samples were analyzed in duplicate and results are demonstrated in pg/mL.

2.6 Data analysis

Data were analyzed using GraphPad Prima software, version 6.01. Cytokine and PGE_2 analyses preceded the normality test (Shapiro Wilk), and groups were compared and analyzed using non-parametric Wilcoxon test. Moments were analyzed using non-parametric Kruskal-Wallis test (cytokine) or Mann Whitney test (PGE_2). For all analysis

$P < 0.05$ was considered to represent significant difference. For gene expression, data are demonstrated descriptively.

3. Results

3.1 Challenge eMSCs with LPS

Cells challenged with LPS at the concentration used allowed realization of the completed assay. Morfology cells modification was not observed at any moment. Additionally, cells grew well without FBS and with no detachment for 24 hours

3.2 Quantitative reverse transcriptase–polymerase chain reaction

Cells reveled expression for all genes studied (Figure 2) at all samples analyzed.

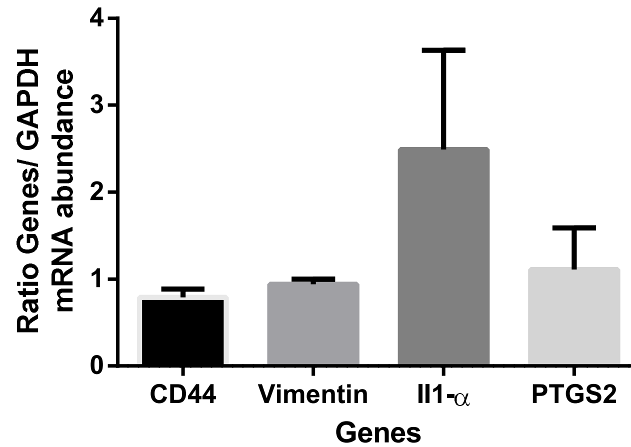


Figure 2: Ratio of genes/GAPDH mRNA abundance measured by quantitative reverse transcriptase–polymerase chain reaction analysis in bovine endometrial mesenchymal stem cells. Results are presented as mediam with interquartile range. IL-1α: interleukin 1α, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, PTGS2: prostaglandin-endoperoxide synthase 2.

3.3 Quantification of Cytokines

Quantification of cytokine reveled production of all cytokines independently of LPS exposure. When groups (Control x Treated) were compared, difference (*P=0.02*) for cytokine TNF-α on moment 6 hours was observed, with greater concentration on treated group samples. When moments from the same group were compared, difference on group control for cytokine IL1-α (Moment 2 hours *versus* 6 hours *P*=0,03; and 6 hours *versus* 12 hours *P*=0,01) was observed (Figure 3 and 4).

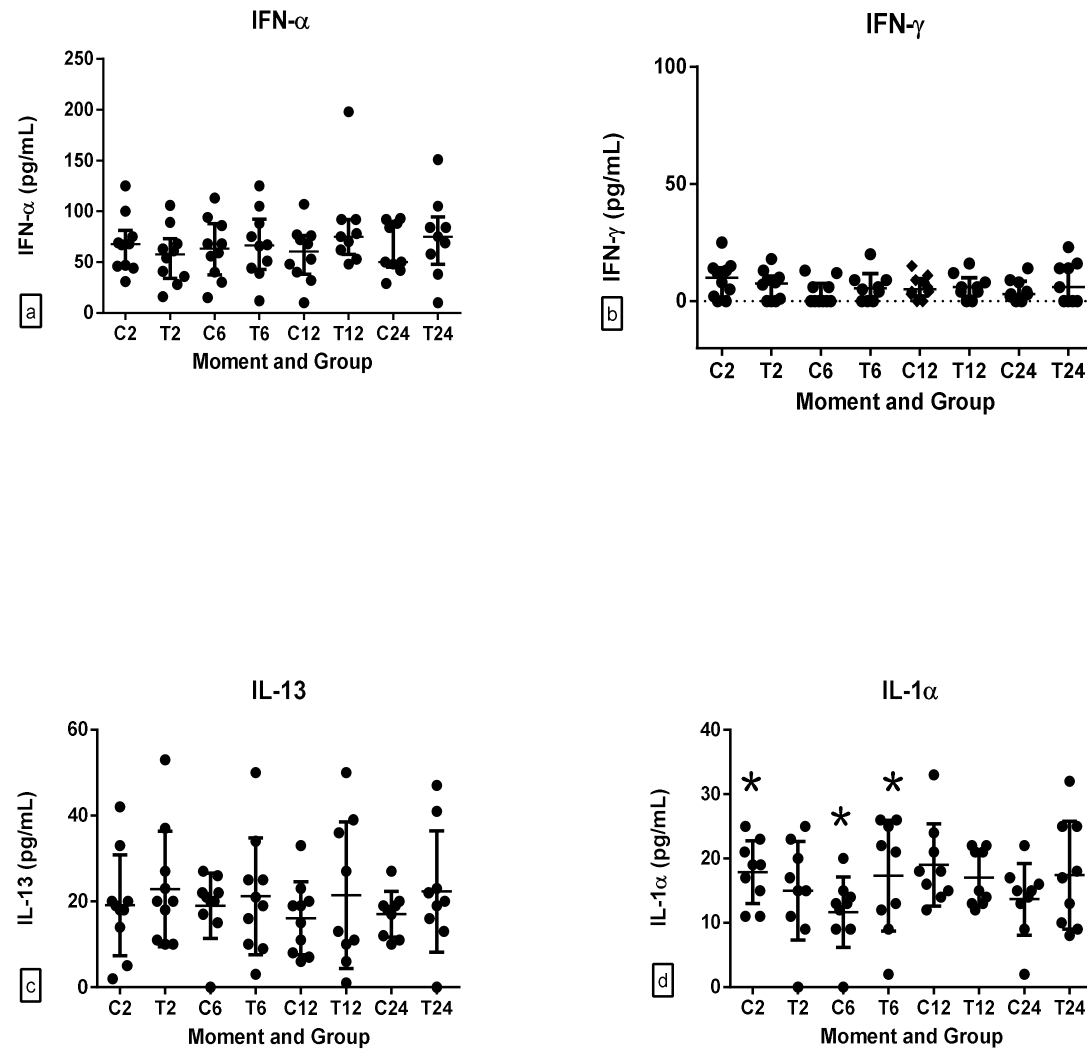


Figure 3: Cytokine concentration of IFN-α (a), INF-γ (b), IL-13 (c) and IL-1α (d) on culture medium of bovine endometrial mesenchymal stem cells challenged or not with LPS, after 2, 6, 12 and 24 hours of exposition. Asterisk (* at IL-1α) represents differences ($P < 0.05$) between moments. C: control, T: treated. Results are presented as mediam with interquartile rage.

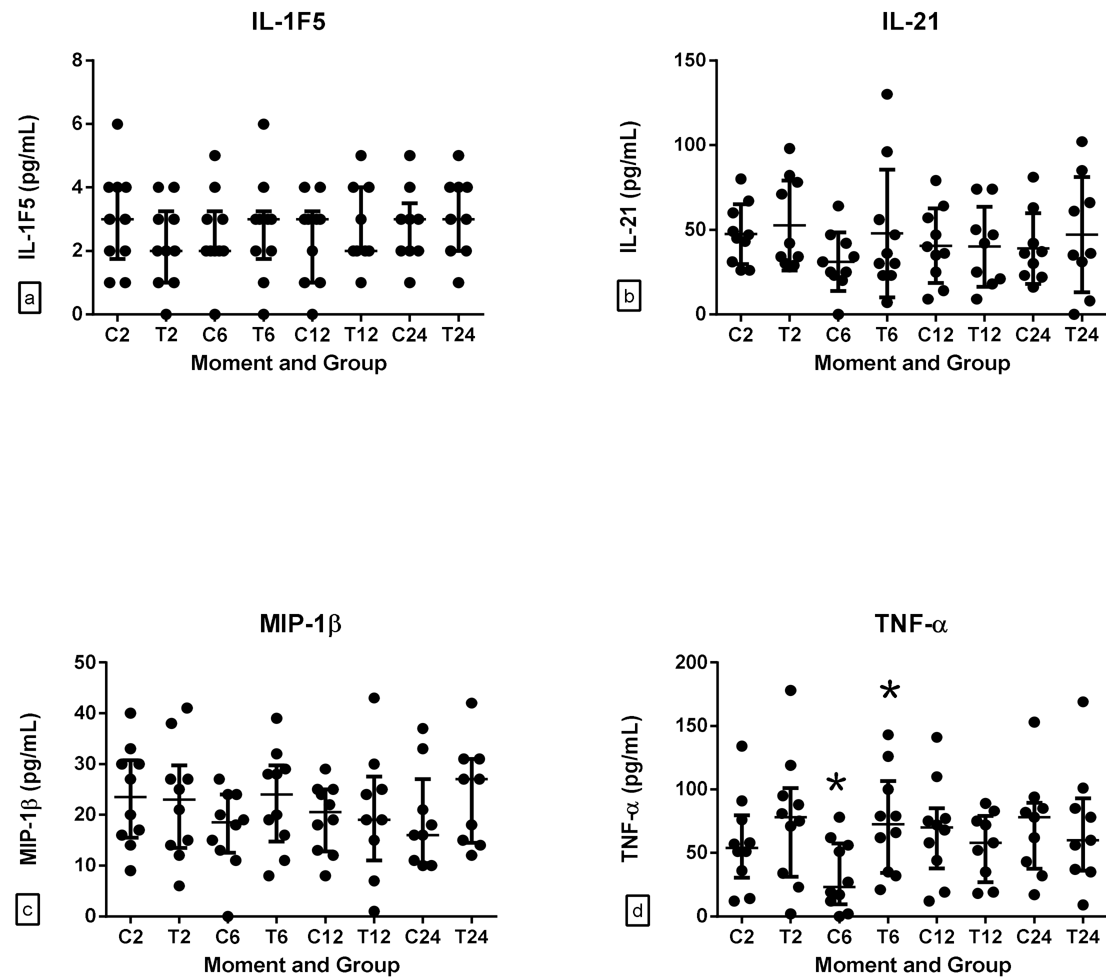


Figure 4: Cytokine concentration of IL-1F5 (a), IL-21 (b), MIP-1β (c) and TNF-α (d) on culture medium of bovine endometrial mesenchymal stem cells challenged or not with LPS, after 2, 6, 12 and 24 hours of exposition. Asterisk (* at TNF-α) represents differences ($P < 0.05$) between groups (control x treated). C: control, T: treated. Results are presented as mediam with interquartile rage.

3.3 Prostaglandin E_2 concentration

It was observed difference on PGE_2 concentration both at moment (2 and 24 hours) and groups (control and treated). There was significant difference (*P=0.008*) between groups on 24 hours and between moments (Moment 2 hours x 24 hours; *P= 0.056*) for treated group. Results are presented at Figure 5.

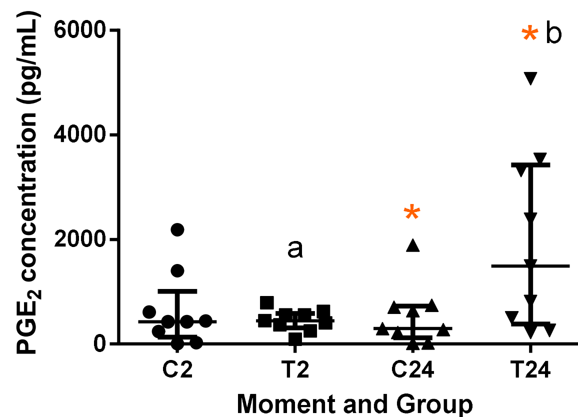


Figure 5: Prostaglandin Ex_2 concentration on bovine endometrial mesenchymal stem cells challenged or not with LPS culture medium after 2 and 24 hours of exposition. Comparison was done between moments and groups. C: control, T: treated. Results are presented as median with interquartile range. Asterisk (*) represents differences (P< 0.05) between groups and different letters represents difference (P< 0.05) between moments.

4. Discussion

It is evident the increasing advancement in the use of MSCs-based therapies and more recently with the CM cell-free for treatment of various conditions and for use at in vitro assays. At the present study, production of important biological molecules

by MSCs related to regulation of immune system was observed. At a proteomic level, proteins related to regulate immune system, tissue remodeling and angiogenesis was already described for bovine eMSCs stimulated or not with LPS [5].

The use of LPS on endometrial cells can be considered to create models of clinical or subclinical endometritis [19], and to test treatments effect [8]. It is known that MSCs are sensitive to culture media and protein profiles may change in response to microenvironments to which they are subjected [20], responding by different mechanisms to defend the organism [5]. It means that MSCs not only have an immunosuppressive effect, but may also secrete cytokines with anti-inflammatory immunosuppressive factors [1], which is desirable once not always immunosuppression effects are favorable [3]. This was observed at our samples in which cells were not exposed to a stimulus, cytokines with anti-inflammatory functions such as IL-13 and IL-1F5 and with pro- inflammatory function such as IFN-γ, IL-1α, IL-21 and TNF-α were found in detectable concentrations. Cytokine production even without a pro-inflammatory factor or stimulus could be inferred to a stress that cells can suffer in the absence of FBS on culture medium, providing the secretion of more biologically active factors. We also emphasize that eMSCs are able to produce substances with immunomodulatory properties after stimulus, and it may the also test at in vivo assays, to verify if cells act in the same way.

Higher concentration of TNF-α, a pro-inflammatory cytokine, at treated group confirmed the response to a LPS stimulus, once it is known that LPS recognition by endometrial cells induces pro-inflammatory production of pro-inflammatory cytokines (such as TNF-α) that induces secretion of proteins with injured tissues remodeling function [21], aiming to minimize organ damage [22]. This high concentration of TNF-α was also observed by Perrini et al. [23] on equine endometrial cells after 3 hours of stimulation with LPS and on bovine endometrial MSCs under no stimulus [24]. Non-detection of differences between groups on another studied cytokines could be

associated with its half-life, sampling time [7] or concentration of LPS which can be deleterious for endometrial cells in vitro, once this effects cannot be modulated as occurs in vivo [23]. Therefore we also can infer that at our experimental conditions, the more appropriate period to study the effect of some therapy using this endometritis model could be at the first six hours, when we observed differences on secretion of cytokines between groups. The difference between concentration at samples (high standard deviation) for the same cytokine bring us important information about the difference in the capacity of different samples

Pure populations of stromal cells are able to produce PGE_2 after stimulus with LPS, by MSCs pro-inflammatory profile activation through a receptor complex TLR-CD14 connection present on cells and LPS [2, 11]. When occurs this activation, MSCs are able to secrete pro-inflammatory factors including NO, IDO and PGE_2 [2]. At the present work, higher concentrations of PGE_2 after LPS stimulus on eMSCs was evidenced after 24 hours. Our results are in accordance with other studies about bovine endometrial cells, that also revealed PGE_2 production in cells exposure to LPS after 1 [8] or 24 hours [11]. Once PGE_2 appears to be the first molecule responsible for the inhibition of lymphocytes proliferation [25] and its immunoregulation by MSCs is done with another immunosuppressive molecule [3], the presence of PGE_2 on an inflammatory microenvironment means to be favorable to control inflammation, preventing an extensive damage on tissue and its repair [2]. With these results, we reinforce that in a pro-inflammatory microenvironment as occurs in cases of endometritis, endometrial cells are able to secrete immunomodulatory molecules to mediate inflammatory cells suppression by MSCs.

Gene expression of MSCs marker CD44 and vimentin was also detected in equine [26], porcine [27] and human [28] endometrial MSCs and are in accordance with the immunophenotypic profile and immunocytochemistry of bovine eMSCs with high levels and positive marking [10]. IL-1α was also found on cytokine analysis, mainly in not stimulated cells. Also, the expression of PTGS2 on these cells was

expected once it participates of PGE_2 synthesis, detected on supernatant of bovine eMSCs even without experience a challenge. On cells exposure to LPS, this PTGS2 have high expression modulated by PGE_2 production [8].

5. Conclusions

Based on these and on our results, we can infer that as bovine eMSCs are able to respond to a stimulus, such which occur in cases of uterine disorders as endometritis, with a production of immunomodulatory substances detected in the conditioned medium, aiming tissue preservation, repair and immunomodulation.

Funding: This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (scholarship Proc.2014/20447-2, Proc. 2015/18964-1 and financial support Proc. 2015/01057-1).

References

[1] D.Kyurkchiev, I. Bochev, E. Ivanova-Todorova, M. Moudjeva, T. Oreschkova, K. Belemezova, S. Kyurchiev, Secretion of immunoregulatory cytokines by mesenchymal stem cells, World J Stem Cells. 26 (2014) 552-570.

[2] M.E Bernardo, W.E. Fibbe, Mesenchymal stromal cells: sensors and switchers on inflammation, Cell Stem Cell. 13 (2013) 1-11.

[3] Y. Shi, J. Su, A.I. Roberts, P. Shou, A.B. Rabson, G. Ren, How mesenchymal stem cells interact with tissue immune responses. Trends Immunol. 33 (2012) 136-143.

[4] C.W. Park, K. Kim, S. Bae, H.K. Son, P. Myung, H.J. Hong, H. Kim, Cytokine secretion profiling of human mesenchymal stem cells by antibody array, International Journal of Stem Cells. 2 (2009) 59-69.

[5] C.N. Moraes, L. Maia, E. Oliveira, C.P. Dell´Aqua, A. Chapwanya, F. Landim-Alvarenga, E. Oba, Shotgun proteomic analysis of the secretome of bovine

endometrial mesenchymal progenitor/stem cells challenged or not with bacterial lipopolysaccharide, Vet. Immuno.l Immunopathol. 187 (2017) 42-47.

[6] S. Heralth, H. Dobson, C.E. Bryant, I.M. Sheldon, Use of the cow as a large animal model of uterine infection and immunity. J Reprod Immunol. 69 (2006) 13–22.

[7] E.J. Williams, D.P. Fischer, D.E. Noakes, G.C.W. England, A. Rycroft, H. Dobson, M. Sheldon, The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology. 68 (2007) 549-559.

[8] M.G. Marine, C. Perrini, P. Esposti, B. Corradetti, D. Bizzaro, P. Riccaboni, E. Fantinato, G. Urbani, G. Gelati, F. Cremonesi, A. Lange-Consiglio, Effects of platelet-rich plasma in a model of bovine endometrial inflammation in vitro, Reprod. Biol. Endocrinol. 14 (2016) 1-17.

[9] L.L. Healy, J.G. Cronin, I.M. Sheldon, Endometrial cells sense and react to tissue damage during infection of the bovine endometrium via interleukin 1, Scientific Reports, 4 (2014) 1-9.

[10] C.N. Moraes, L. Maia, M.D. Camargos, C.P. Freitas-Dell Aqua, L.S.L.S. Mota, A. Chapwanya, F. C. Landim-Alvarenga, E. OBA, Bovine endometrial cells: a promising source of mesenchymal stem/progenitor cells, Cell Biol. Int. 40 (2016) 1332-1339.

[11] S. Heralth, S.T. Lilly, D.P. Fischer, E.J. Williams, H. Dobson, C.E. Bryant, M. Sheldon, Bacterial lipopolysaccharide induces an endocrine switch from Prostaglandin $F_{2\alpha}$ to prostaglandin E_2 in bovine endometrium, Endocrinology. 150 (2009) 1912-1920.

[12] J.J. Ireland, R.L. Murphee, P.B.Coulson, Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum, J. Dairy Sci.63 (1980) 155-160.

[13] A. Chapwanya, K.G. Meade, M. Doherty, J.J. Callanan, C. O´Farrelly, Endometrial epithelial cells are potent producers of tracheal antimicrobial peptide and

serum amyloid A3 gene expression in response to E.coli stimulation, Vet. Immunol. Immunopathol. 151 (2013) 157-162.

[14] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e.45.

[15] M. Pashaiasl, K. Khodadadi, M.K. Holland, P.J. Verma, The efficient generation of cell Lines from bovine parthenotes, Cell Reprogram. 12 (2010) 571-579..

[16] E.S. Caixeta, M.L. Sutton-McDowall, R.B. Gilchrist, J.G. Thompson, C.A. Prime, M.F. Machado, P.F. Lima, J. Buratini, Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes, Reproduction, 146 (2013) 27-35.

[17] S. Konnai, T. Usui, K. Oshashi, M.Onuma, The rapid quantitative analysis of bovine cytokine genes by real-time RT-PCR, Vet. Microbiol. 94 (2003) 283-294.

[18] C.N. Moraes, L. Maia, P.F Lima, M.C. Dias, T.M.M. Raposo-Ferreira, M.J. Sudano, J.B. Junior, E. Oba, Temporal analysis of prostaglandin F2a receptor, caspase 3, and cyclooxygenase 2 messenger RNA expression and prostaglandin F2a receptor and cyclooxygenase 2 protein expression in endometrial tissue from multiparous Nelore (Bos taurus indicus) cows treated with cloprostenol sodium during puerperium, Theriogenology. 83 (2015) 276-284.

[19] D. Salilew-Wondim, S. Ibrahim, S. Gebremedhn, D. Tesfaye, M. Heppelmann, H. Bollwein, C. Pfarrer, E. Tholen, C. Neuhoff, K. Schellander, M. Hoelker, Clinical and subclinical endometritis induced alterations in bovine endometrial transcriptome and miRNome profile, BMC Genomics. 17 (2016) 218-239.

[20] J.R. Lavoie, M. Rosu-Myles, Uncovering the secretes of mesenchymal stem cells, Biochimie. 95 (2013) 2212–2221.

[21] H. Baumann, J. Gauldie, The acute phase response, Immunol. Today. 15 (1994) 74–80.

[22] L. Zachar, D. Bacenková, J. Rosocha, Activation, homing, and role of the mesenchymal stem cells in the inflammatory environment, J. Inflamm. Res. 9 (2016) 231-240.

[23] C. Perrini, M.G. Strillacci, A. Bagnato, P. Esposti, M.G. Marini, B. Corradetti, D. Bizzaro, A. Idda, S. Ledda, E. Capra, F. Pizzi, A. Lange-Consiglio, F. Cremonesi, Microvesicles secreted from equine amniotic-derived cells and their potential role in reducing inflammation in endometrial cells in an in-vitro model, Stem Cell Res. Ther. 7 (2016), 169-184.

[24] G. Donofrio, V. Franceschi, A. Capocefalo, S. Cavirani, I.M. Sheldon, Bovine endometrial stromal cells display osteogenic properties, Reprod. Biol. Endocrinol. 6 (2008) 1–9.

[25] D.D. Carrade, D.L. Borjesson, Immunomodulation by Mesenchymal Stem Cells in Veterinary Species, Comp. Med. 63 (2013) 207-217.

[26] B.E. Rink, K.R. Amilon, C.L. Esteves, H.M. French, E. Watson, C. Aurich, F.X. Donadeu, Isolation and characterization of equine endometrial mesenchymal stromal cells, Stem Cell Res. Ther. 8 (2017) 1 66- 178.

[27] K. Miernik, J. Karasinski, Porcine uterus contains a population of mesenchymal stem cells, Reproduction 143 (2012) 203–239.

[28] C.E. Gargett, K.E. Schwab, R.M. Zilwood, H.P.T. Nguyen, D. Wu, Isolation and culture of epithelial progenitors and mesenchymal stemcells from human endometrium, Biol. Reprod. 80 (2009) 1136–45.

CAPÍTULO 4

7. CONSIDERAÇÕES GERAIS

De acordo com os objetivos propostos, e nas condições experimentais podemos concluir que:

- As CTMsE de bovinos nas fases II e III do ciclo estral foram isoladas e cultivadas com sucesso, sem diferença entre as mesmas;

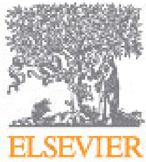
- As CTMsE de bovinos nas fases II e III do ciclo estral apresentaram baixa imunogenicidade (ausência de expressão de MHC-II), elevada expressão de marcadores de superfície comumente preditos para CTMs (CD44, CD29), ampla capacidade de formação de colônias fibroblásticas, estabilidade cromossômica em cultivo e habilidade para diferenciar em linhagens mesodermais (multipotencialidade) independente da fase estudada;

- As CTMsE de bovinos nas fases II e III do ciclo estral apresentam taxas de viabilidade satisfatórias após a criopreservação independente do meio utilizado. Adicionalmente, a ausência de diferenças entre os meios de criopreservação nos permite inferir que o meio condicionado, com menor concentração de SFB (imunogênico) na composição possa ser uma boa alternativa à criopreservação deste tipo celular;

- A avaliação do secretoma das CTMsE de bovinos por análise proteômica através do seu meio condicionado permitiu a identificação nos grupos expostos ou não ao LPS bacteriano de proteínas de interesse e com amplo potencial terapêutico.

- As CTMsE de bovinos produzem maior quantidade de citocinas e prostaglandina E_2 após estímulo com LPS bacteriano.

ANEXOS



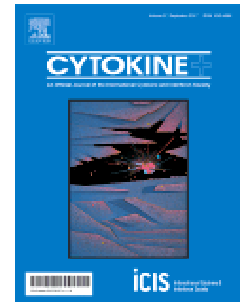
CYTOKINE

An Official journal of the International Cytokine & Interferon Society (ICIS)

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

- **Description** **p.1**
- **Impact Factor** **p.1**
- **Abstracting and Indexing** **p.2**
- **Editorial Board** **p.2**
- **Guide for Authors** **p.4**



ISSN: 1043-4666

DESCRIPTION

* Devoted exclusively to the study of the **molecular biology**, **genetics**, **biochemistry**, **immunology**, **genome-wide association studies**, **pathobiology**, diagnostic and clinical applications of all known **interleukins**, **hematopoietic** factors, growth factors, **cytotoxins**, **interferons**, new **cytokines**, and **chemokines**, *Cytokine* provides comprehensive coverage of cytokines and their mechanisms of actions, 12 times a year by publishing original high quality refereed scientific papers from prominent investigators in both the academic and industrial sectors.

We will publish 3 major types of manuscripts:
1) Original manuscripts describing research results.
2) Basic and clinical reviews describing cytokine actions and regulation.
3) Short commentaries/perspectives on recently published aspects of cytokines, pathogenesis and clinical results.

Special issues:
Suggestions for special issue topics and guest editors can be sent to Jenny Henzen, j.henzen@elsevier.com

Benefits to authors
We also provide many author benefits, such as free PDFs, a liberal copyright policy, special discounts on Elsevier publications and much more. Please click here for more information on our author services .

Please see our Guide for Authors for information on article submission. If you require any further information or help, please visit our Support Center

IMPACT FACTOR

2016: 3.488 © Thomson Reuters Journal Citation Reports 2017

ABSTRACTING AND INDEXING

BIOSIS
Chemical Abstracts
Current Contents
MEDLINE®
EMBASE
Science Citation Index
Scopus

EDITORIAL BOARD

Editor:

D. Kalvakolanu, University of Maryland School of Medicine, Baltimore, Maryland, USA

Associate Editors:

A. Diefenbach, Charité - Universitatsmedizin Berlin, Berlin, Germany
S.L. Gaffen, University of Pittsburgh, Pittsburgh, Pennsylvania, USA
G. Gallagher, HUMIGEN: The Institute for Genetic Immunology, Hamilton, New Jersey, USA
B. Jenkins, National Health and Medical Research Council (NHMRC), Clayton, Victoria, Australia
K. Mossman, McMaster University, Hamilton, Ontario L8S 4K1, Canada
K.D. Moudgil, University of Maryland School of Medicine, Baltimore, USA
N. Mukaida, Kanazawa University, Kanazawa, Japan
T.B. Niewold, Mayo Clinic, Rochester, Minnesota, USA
R. Thorpe, National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK
Z. Tian, University of Science and Technology of China (USTC), Hefei, Anhui, China

Founding Editors:

Sir Gordon W. Duff, University of Sheffield, Sheffield, UK
S.K. Durum, National Cancer Institute (NCI), Frederick, Maryland, USA

Editorial Board:

S. Balachandran, Philadelphia, Pennsylvania, USA
P.S. Biswas, Pittsburgh, Pennsylvania, USA
M. Cassatella, Verona, Italy
B. Damania, Chapel Hill, North Carolina, USA
S. Dhib-Jalbut, New Brunswick, New Jersey, USA
C.A. Dinarello, Denver, Colorado, USA
S.Y. Fuchs, Philadelphia, Pennsylvania, USA
C. Gabay, Geneva 14, Switzerland
M. Gale (Jr.), Seattle, Washington, USA
A. Gamero, Philadelphia, Pennsylvania, USA
J Hiscott, Rome, Italy
L.A.B. Joosten, Nijmegen, Netherlands
A. Keegan, Baltimore, Maryland, USA
A. Khaled, Orlando, Florida, USA
E. Lubberts, Rotterdam, Netherlands
A. Mantovani, Milan, Italy
M. McGeachy, Pittsburgh, Pennsylvania, USA
N. Mehta, Bethesda, Maryland, USA
G. Meng, Shanghai, China
M.S. Miller, Hamilton, Ontario, Canada
P. Miossec, Lyon, France
J.D. Mountz, Birmingham, Alabama, USA
M. Mueller, Vienna, Austria
S. Mustjoki, Helsinki, Finland
L. O'Neill, Dublin 2, Ireland
S. Paludan, Aarhus, Denmark
J.-H. Park, Bethesda, Maryland, USA
D. Parker, New York, New York, USA
A. Proudfoot, Plan les Ouates, Geneva, Switzerland
T. Putoczki, Parkville, Victoria, Australia
R.T. Robinson, Milwaukee, Wisconsin, USA
S. Rose-John, Kiel, Germany
B. Saha, Pune, India

R. Savan, Seattle, Washington, USA
S. Sozzani, Brescia, Italy
T. Tanaka, Osaka, Japan
H.R. Turnquist, Pittsburgh, Pennsylvania, USA
F. L. van der Veerdonk, Nijmegen, The Netherlands
N Ward, Cleveland, Ohio, USA
H. Yoshida, Saga, Japan
C. Zhang, Jinan, China
J. Zucman-Rossi, Paris, France

GUIDE FOR AUTHORS

Your Paper Your Way

We now differentiate between the requirements for new and revised submissions. You may choose to submit your manuscript as a single Word or PDF file to be used in the refereeing process. Only when your paper is at the revision stage, will you be requested to put your paper in to a 'correct format' for acceptance and provide the items required for the publication of your article.
To find out more, please visit the Preparation section below.

INTRODUCTION

The official journal of the *International Cytokine Society*

Cytokine is devoted exclusively to the study of the molecular biology, biochemistry, immunology, diagnostic and clinical applications of all known interleukins, hematopoietic factors, growth factors, cytotoxins, interferons, and new cytokines, *Cytokine* provides comprehensive coverage of cytokines and their receptors, 12 times a year, by publishing original high quality refereed scientific papers from prominent investigators in both the academic and industrial sectors.

Types of paper

Review Articles are comprehensive appraisals of research and clinical outcomes in a field of current interest related to cytokine biology. All reviews are subject to the normal peer review process. Reviews are mostly invited by the *Reviews Associate Editor* but interested authors may contact Dr. Sarah Gaffen at sig65@pitt.edu to discuss ideas or present an outline.

Research Articles are full-length descriptions of original research. The scope may include basic science, clinical results, or applications. These manuscripts will undergo standard review.

Short Communications. These submissions will also undergo standard review. Manuscripts should not exceed 2000 words plus no more than 15 references. Your cover letter must include your word count. Results and Discussion sections may be combined. No more than 2 Figures and/or Tables should be included.
Cytokine Stimulus Cytokine stimulus articles describe significance and how it explains or contradicts the reported literature. Manuscripts should not exceed 1500 words plus one figure and a maximum of 10 references.

CYTOKINE policy on papers describing Genetic Polymorphisms All SNP (single nucleotide polymorphism) analyses must include at least 400 individual samples. SNP data must be accompanied with the measurement of specific immunological parameters such as cytokine measurement, flow cytometry, cellular responses, etc., demonstrating a comparison of biological data between subjects with the genetic variant and those who lack the genetic variant being studied. Expression of cell surface receptors in the cells of immune system by flow coupled with genetics of that receptor, or looking at TH1/TH2 skewing or cellular maturation in the setting of particular variants are recommended. Papers lacking such data will not be considered for review.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
• E-mail address
• Full postal address

All necessary files have been uploaded:
Manuscript:
• Include keywords
• All figures (include relevant captions)
• All tables (including titles, description, footnotes)
• Ensure all figure and table citations in the text match the files provided
• Indicate clearly if color should be used for any figures in print

Graphical Abstracts / Highlights files (where applicable)
Supplemental files (where applicable)

Further considerations
• Manuscript has been 'spell checked' and 'grammar checked'
• All references mentioned in the Reference List are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Internet)
• A competing interests statement is provided, even if the authors have no competing interests to declare
• Journal policies detailed in this guide have been reviewed
• Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

BEFORE YOU BEGIN

Ethics in publishing
Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Human and animal rights
If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals. Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed.

Declaration of interest
All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state this: 'Conflicts of interest: none'. More information.

Submission declaration and verification
Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see 'Multiple, redundant or concurrent publication' section of our ethics policy for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck.

Authorship
All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Changes to authorship
Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason

for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.
Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Article transfer service
This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. Please note that your article will be reviewed again by the new journal. More information.

Copyright
Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (see more information on this). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

For open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (more information). Permitted third party reuse of open access articles is determined by the author's choice of user license.

Author rights
As an author you (or your employer or institution) have certain rights to reuse your work. More information.

Elsevier supports responsible sharing
Find out how you can share your research published in Elsevier journals.

Role of the funding source
You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies
Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some funding bodies will reimburse the author for the Open Access Publication Fee. Details of existing agreements are available online.

Open access
This journal offers authors a choice in publishing their research:

Open access
• Articles are freely available to both subscribers and the wider public with permitted reuse.
• An open access publication fee is payable by authors or on their behalf, e.g. by their research funder or institution.

Subscription
• Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs.
• No open access publication fee payable by authors.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution (CC BY)
Lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)
For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The open access publication fee for this journal is **USD 3300**, excluding taxes. Learn more about Elsevier's pricing policy: https://www.elsevier.com/openaccesspricing.

Green open access
Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our green open access page for further information. Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form. Find out more.

This journal has an embargo period of 12 months.

Language (usage and editing services)
Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

Submission
Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Submission address
Please submit your article via http://ees.elsevier.com/ycyto

Referees
Please submit, with the manuscript, the names and addresses (including contact email addressess) of a minimum of 5 people suggested as reviewers for your manuscript. Please include reviewers who are independent and unbiased, from different institutions. You may also mention persons who you would prefer not to review your paper.

PREPARATION

NEW SUBMISSIONS
Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process.
As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay-out that can be used by referees to evaluate your manuscript. It should contain high enough quality

figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements
There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.
If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.
Divide the article into clearly defined sections.

Figures and tables embedded in text
Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure or table.

Peer review
This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of one independent expert reviewer to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

REVISED SUBMISSIONS
Use of word processing software
Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure
Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract
Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site.
Authors can make use of Elsevier's Illustration Services to ensure the best presentation of their images and in accordance with all technical requirements.

Highlights
Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Nomenclature and units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI. You are urged to consult IUPAC: Nomenclature of Organic Chemistry for further information.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files.

A detailed guide on electronic artwork is available.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
• Supply files that are too low in resolution.
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

Illustration services
Elsevier's WebShop offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical-style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Figure captions
Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables
Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References
Responsibility for the accuracy of bibliographic citations lies entirely with the Authors.

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Data references
This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software
Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley and Zotero, as well as EndNote. Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their

article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:
http://open.mendeley.com/use-citation-style/cytokine
When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13.03.03).
Reference to a dataset:
[dataset] [5] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/xwj98nb39r.1.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations.

Video
Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 150 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary material
Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

RESEARCH DATA
This journal encourages and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page.

Data linking
If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving readers access to underlying data that give them a better understanding of the research described.

There are different ways to link your datasets to your article. When available, you can directly link your dataset to your article by providing the relevant information in the submission system. For more information, visit the database linking page.

For supported data repositories a repository banner will automatically appear next to your published article on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

Data in Brief
You have the option of converting any or all parts of your supplementary or additional raw data into one or multiple data articles, a new kind of article that houses and describes your data. Data articles ensure that your data is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. You are encouraged to submit your article for *Data in Brief* as an additional item directly alongside the revised version of your manuscript. If your research article is accepted, your data article will automatically be transferred over to *Data in Brief* where it will be editorially reviewed and published in the open access data journal, *Data in Brief*. Please note an open access fee of 500 USD is payable for publication in *Data in Brief*. Full details can be found on the Data in Brief website. Please use this template to write your Data in Brief.

AudioSlides
The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Interactive plots
This journal enables you to show an Interactive Plot with your article by simply submitting a data file. Full instructions.

AFTER ACCEPTANCE

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

AUTHOR INQUIRIES

Visit the Elsevier Support Center to find the answers you need. Here you will find everything from Frequently Asked Questions to ways to get in touch.
You can also check the status of your submitted article or find out when your accepted article will be published.

© Copyright 2014 Elsevier | http://www.elsevier.com

Cell Biology International ISSN 1065-6995
doi: 10.1002/cbin.10688

RESEARCH ARTICLE

Bovine endometrial cells: a source of mesenchymal stem/progenitor cells

Carolina Nogueira de Moraes[1]*, Leandro Maia[1], Marianne Camargos Dias[1], Camila P. Freitas Dell'Aqua[1], Ligia Souza Lima Silveira da Mota[2], Aspinas Chapwanya[3], Fernanda da Cruz Landim-Alvarenga[1] and Eunice Oba[1]

1 Department of Animal Reproduction and Radiology, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil
2 Department of Genetics, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil
3 Department of Clinical Sciences, Ross University School of Veterinary Medicine, Basseterre, St Kitts and Nevis

Abstract

Endometrial mesenchymal stem/progenitor cells (eMSCs) are multipotent cells known to modulate the immune system and have clinical application for human and animal health. This makes these bovines cells attractive for dual use as cellular therapy and experimental model. The aim of this study was to isolate, evaluate the differentiation potential, immunophenotypic and immunocytochemistry characteristics, chromosomal stability, cloning efficiency, and cryopreservation response of bovine eMSCs collected in two phases of the estrous cycle. For this, cells were isolated and submitted to differentiation for adipogenic and osteogenic lineage. The cells were then characterized by flow cytometer (FC) (vimentin, CD29, CD44, MHC-II, CD34) and immunocytochemistry (vimentin, pan-cytokeratin, CD44) and submitted to cytogenetic and cloning efficiency assay. The cells were also cryopreserved using two different medium of cryopreservation and analyzed by FC for viability, necrosis, late-apoptosis + necrosis, and initial apoptosis rates before and after cryopreservation. We obtained homogeneous cell populations which have fibroblastic morphology and adherence to plastic. These cells expressed high levels of markers CD29, CD44, and vimentin, low expression levels for CD34 and no MHC-II. The cells were chromosomally stable ($2n = 60$) with high cloning efficiency and no difference ($P > 0.05$) between medium of cryopreservation or phase was observed after thawing. We showed the presence and differentiation potential of bovine eMSCs, with chromosomal stability and great response to cryopreservation with both medium, which has implications for build biobanks or development of new therapeutic approaches to combat uterine diseases or to study.

Keywords: characterization; cow; cryopreservation; endometrium; progenitor cells

Introduction

Mesenchymal stem/progenitor cells (MSCs) are multipotent cells which have attracted great interest in the fields of human and animal medicine because of their unique immunomodulatory properties. Many studies have described the feasibility of these cells as therapeutics because of their antiapoptotic and self-renewal properties. Specifically, these cells are known to downregulate immune responses by promoting regulatory T cells and inhibit cytotoxic T cell proliferation (Stenger et al., 2015).

Bovines are a suitable experimental model and have several advantages for use in clinical studies on application of MSCs for human medicine (Bosnakovski et al., 2004). The cells can readily be obtained from endometrial tissue and expanded in vitro. In addition, it is possible to acquire large tissue samples for biobanking cells with good viability for future use in regenerative medicine.

The endometrial tissue is a highly regenerative tissue and contains endometrial stromal cells, which are dynamic, and have the capacity for growth and differentiation during the estrous cycle and pregnancy in cows (Donofrio et al., 2008).

*Corresponding author: e-mail: carolnmoraes@hotmail.com

Abbreviations: AN, annexin V; CE, cloning efficiency; CD, cluster of differentiation; CFU-F, fibroblastic colony-forming unit assay; CL, corpus luteum; DMEM, Dulbecco's Modified Eagle's Medium; eMSCs, endometrial mesenchymal stem/progenitor cells; FBS, fetal bovine serum; FC, flow cytometer; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; MHC, major histocompatibility complex; MSCs, mesenchymal stem/progenitor cells; PI, propidium iodide; SEM, standard error of the mean

 © 2016 International Federation for Cell Biology

The presence of endometrial MSCs (eMSCs) has been described in other mammals such as human (Garget et al., 2015), pig (Miernik and Karasinski, 2012), ovine (Letouzey et al., 2015), and mouse (Chan and Gargett, 2006).

MSCs are a viable alternative in the treatment of endometrial degeneration in humans (Meng et al., 2007), endometrosis (Mambelli et al., 2013), and fibrosis (Alvarenga et al., 2016) in mares. In humans, eMSCs or menstrual blood MSCs were used in cardiac (Bockeria et al., 2013), neurological (Wolff et al., 2011), and models of metabolic diseases (Santamaria et al., 2011) showing their great application. In non-primate animals which do not undergo menstruation, eMSCs participate in endometrial remodeling after each estrus event, and also in the regeneration of endometrial stroma during the postpartum period (Letouzey et al., 2015). Thus, these cells may have a direct application in treatment of reproductive pathologies that require an endometrial remodeling such as endometritis and fibrosis. Only a few studies describe in detail the presence of these cells in bovines, and even fewer give an in-depth characterization of these cells, which would be of great interest in clinical treatments.

To our knowledge, there is no study on chromosomal stability or potential of cryopreservation of bovine eMSCs which reinforce the possibility of the creation of biobanks for future therapeutic use. So, the purpose of this study was to isolate, culture, and characterize MSCs obtained from the bovine endometrium using the techniques of flow cytometry, immunocytochemistry, cloning efficiency, and cytogenetics. In addition, we evaluated the response to cryopreservation using two medium of cryopreservation. These cells were collected and compared at two phases of the estrous cycle, and our findings can contribute for a better knowledge of biological properties and therapeutic potential of bovine MSCs.

Material and methods

The study was performed according to the ethical guidelines recommended by the National Council for Control of Animal Experimentation and the College of Animal Experimentation, and it was approved by the institution's Animal Care and Experimentation Ethics Committee (Protocol Number 152/2014).

Selection of endometrial material

The uteri and ovaries of non-pregnant cows ($n = 12$) were selected at a local slaughterhouse immediately after slaughter and kept on ice until processing in the laboratory. The physiological statuses of the tracts were determined by observation of uterine and ovarian structures, particularly the corpus luteum (CL) according to methodology described by Ireland et al. (1980) and Chapwanya et al. (2013). All the animals selected were estrous cycling (Phase II; $n = 6$/Phase III; $n = 6$), with no evidence of uterine disease.

Processing, isolation, and culture of MSCs

The uterine horn ipsilateral to the CL corresponding to each phase was sectioned and kept in 70% alcohol solution for 2 min (Chapwanya et al., 2013). Subsequently, the endometrial tissue was separated from myometrial tissue with the aid of a sterile scissors, and washed with wash solution composed of HBSS solution (50 mL) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (3 μg/mL) (Thermo Fisher Scientific®, USA) and amikacin (22 μg/mL) (Teuto®, BRA).

The endometrial tissue was cut into small pieces and submitted to digestion performed in two steps (Fortier et al., 1988 with modifications). The first digestion was done using 0.3% trypsin (Sigma®, USA) in solution of 10 mL of HBSS (Thermo Fisher Scientific®) for 3 h at 22°C under agitation. Subsequently, the tissue was washed with wash solution and filtered in 40 μm filter (Becton Dickinson and Company®, USA). The second digestion was done with a solution composed of 0.5 mg/mL of trypsin, 0.5 mg/mL of collagenase, 1 mg/mL of bovine serum albumin, and 0.1 mg/mL of DNase I (Sigma®) in HBSS (Thermo Fisher Scientific®) for 1.5 h at 37°C. After filtration in 40 μm filter, the material was resuspended in HBSS medium with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific®) and centrifuged (Hermle centrifuged Z200A, GER) twice at 100×*g*/10 min.

After viability assay with Tripan blue solution 0.4% (Gibco®, USA), the material was plated (1.25×10^5 cell/24-well plate) and cultured in medium comprising DMEM high glucose/F12 (1:1), 20% FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (3 μg/mL) (Thermo Fisher Scientific®), and amikacin (11 μg/mL) (Teuto®) at 37.5°C in humid atmosphere containing 95% air and 5% CO_2. The first change of medium occurred 18 h after to remove the epithelial cells (Fortier et al., 1988) and additionally the differential trypsinization (Anand et al., 2012) with 0.125% of trypsin at 0.05% (Sigma®) was performed to aid in the purification of samples on subsequent passages.

Immunophenotypic analysis

Immunophenotypic analyses were performed on cells in the third passage (Phase II; $n = 5$/Phase III; $n = 5$).

For the analysis, the following antibodies were used: mouse anti-vimentin (clone V9, MCA862, AbD Serotec-Rad®, UK), mouse anti-CD29:Alexa fluor 647 (clone TS2/16, 303007, BioLegend®, USA), mouse anti-bovine CD44:FITC (clone IL-A118, MCA2433F, AbD Serotec®,

UK), mouse anti-horse MHC-II:FITC (clone CVS20, MCA1085F, AbD Serotec®, UK) and rabbit anti CD-34: FITC (polyclonal, orb247244, Biorbyt®, USA). For the antibody mouse anti-vimentin cells were previously fixed and permeabilized with Cytofix/Cytoperm™ (Becton Dickinson and Company®) and incubated with the secondary goat-anti mouse:FITC (ab7064, abcam®, USA). The acquisition was made by Flow LSR Fortessa equipment (BD Biosciences, USA) and the analysis was done using the BD FACSDiva™ software (BD Biosciences). During the analysis were accounted for 10,000 events.

Immunocytochemistry characterization

Immunocytochemistry analysis was done in third passage (Phase II; n = 3/Phase; III n = 3) on 24 well-plates (Sarstedt®, USA) according to methodology already described by our group (Maia et al., 2013). The antibodies tested were mouse-anti CD44 (1:100, clone BAG40A, WS0507B-100, VMRD®, USA), mouse anti-vimentin (1:200, clone V9, M0725, Dako®, UK), and mouse anti-pan-cytokeratin (1:100, clone AE1/AE3, M3515, Dako®, USA) with reactivity proven for the species for the last two (Souza et al., 2014). After detection and revelation, the evaluation of the reaction was performed in an inverted light microscope, with a digitizer (LeicaVR Microsystems®, GERM).

Assays for differentiation

The differentiation assay was done for adipogenic and osteogenic (Phase II; n = 6/Phase III; n = 6) lineages with cells in third passage. For this, cells were plated in 6-wells (5200 cells/cm^2) or 24 wells (25000 cells/cm^2) plates for adipogenic and osteogenic differentiation, respectively. After 60% confluence, the differentiation medium was added to the subcultures in triplicate, according to manufacturer's recommendations (Thermo Fisher Scientific®) added by 5% rabbit serum (Maia et al., 2013) or 20% FBS (Toupadakis et al., 2010) at adipogenic and osteogenic medium, respectively.

The confirmation of the osteogenic and adipogenic differentiation was performed, respectively, after 14 and 8 days by demonstrating the deposition of calcium matrix using the histological staining method Alizarin red pH 4.2 (Sigma®) and the presence of intracytoplasmic fat droplets using the dye Oil red 0.5% in alcohol isopropyl (Sigma®).

Cytogenetic analysis

Cytogenetic analysis was performed on samples (Phase II; n = 6/Phase III; n = 6) at first passage using the synchronization technique of cellular cycle by the method of methotrexate/thymidine/colchicine (0.0016%) (Maia et al., 2013).

Fibroblastic colony-forming unit assay (CFU-F) for determination of cloning efficiency (CE)

CFU-F assay (Phase II; n = 5/Phase III; n = 5) was made with cells on second passage according to Mensing et al. (2011) with modifications. For this, cells were seeded (105 cells/cm^2) in 6-well plates, in triplicate. The exchange of medium was done with 72 h and at the 5° day cultures were fixed and stained with crystal-violet in 100% methanol. Colonies with more than 20 cells were accounted and the CE was done using the formula = (counted colonies/cells seeded) × 1,000.

Cryopreservation

The bovine eMSCs from both phases were cryopreserved at a final concentration of 1×10^6 cell/cryotube with the following medium of cryopreservation: Medium 1 (MI): 90% FBS (Thermo Fisher Scientific®), 10% dimethyl sulfoxide (DMSO) (Sigma®), penicilin (100 UI/mL), streptomycin (100 μg/mL), amphotericin B (3 μg/mL); Medium 2 (MII): 90% conditioned medium (DMEM high glucose/F12 (1:1), 20% FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (3 μg/mL) (Thermo Fisher Scientific®), and amikacin (11 μg/mL) (Teuto®), 10% DMSO (Sigma®).

Samples were cryopreserved at a controlled cryogenic container (Mr. Frosty, Nalgene, 5100-001), maintained on a −80°C freezer (Thermo Fisher Scientific®) for 24 h and conserved on liquid nitrogen for 1 month. After this period, cells were thawed and samples cryopreserved with MI and MII (Phase II; n = 5 / Phase III; n = 5) were evaluated by FC.

The FC analyses were done before and after cryopreservation using annexin V APC (AN, Becton Dickinson® and Company, USA) and propidium iodide (PI, Sigma®) for viability (PI− AN−), necrosis (PI+ AN−), late-apoptosis + necrosis (PI+ AN+), and initial apoptosis (PI− AN+) (Ranera et al., 2012).

Data analysis

Data from differentiation assays and immunocytochemistry were presented descriptively and compared qualitatively during the phases (II and III). The other variables with normal distribution were analyzed by t-test or submitted to the non-parametric Mann-Whitney rank sum test at the SigmaPlot software (version 11.0, 2008), adopting $P < 0.05$ as significant. The results were presented as mean and standard error of the mean (SEM).

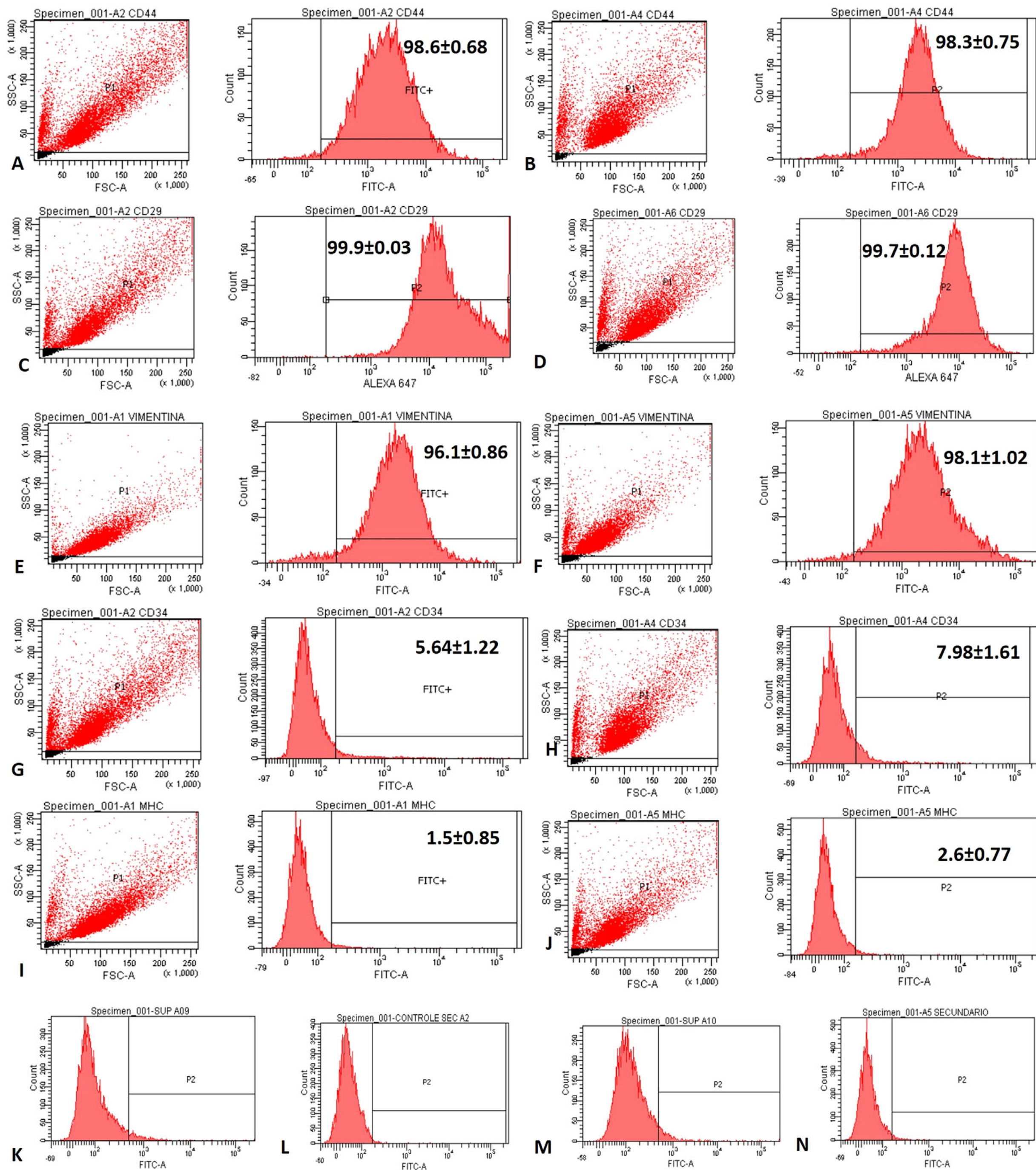


Figure 1 Immunophenotypic analysis for the markers CD44, CD29, vimentin, CD34, and MHC-II from samples of bovine eMSCs from Phase II (A, C, E, G, I) and Phase III (B, D, F, H, J). Representative histograms of marker CD44 from Phase II (A) and III (B). Representative histograms of marker CD29 from Phase II (C) and III (D). Representative histograms of marker vimentin from Phase II (E) and III (F). Representative histograms of marker CD34 from Phase II (G) and III (H). Representative histograms of marker MCH-II from Phase II (I) and III (J). Representative histograms of isotype control IgG from Phase II (K) and III (M). Representative histograms of secondary control from Phase II (L) and III (N). Data are presented as mean and SEM. There were no differences between groups ($P > 0.05$).

Results and discussion

At the present study, we observed results were very interesting and promising in bovine eMSCs independent of the phase of estrous cycle studied. These findings mainly include the high expression of stem/progenitor cells markers (CD29 and C44), low immunogenicity (no expression of MHC-II), chromosomal stability ($2n = 60$) excellent clonicity, differentiation potential on mesodermal lineages, as well as excellent resistance to cryopreservation demonstrated by the good post-thaw viability. These are important and desirable characteristics for the establishment of cells biobank with progenitor cells lines standardized for future use in cell therapy and/or further use for in vitro studies.

Here, the bovine eMSCs at the two estrous cycle phases (II and III) were adherent to plastic and had fibroblastoid morphology within 6 h of culture. Immunophenotypic profile by FC of bovine eMSCs revealed high expression for CD29, CD44, and vimentin, low expression for CD34 and no expression for MHC-II, with no difference ($P > 0.05$) between phases (Figure 1). We emphasize that the threshold used for negative expression was greater than 3%. Qualitative immunocytochemistry analysis of bovine eMSCs revealed positive staining for CD44, vimentin, and pancytokeratin (Figure 2) with the same pattern of staining at both phases.

Unlike human, which counts with a panel of specific surface markers (Dominici et al., 2006), specific surface markers for bovine eMSCs are not well-known and much less established. The significant marking in both immunophenotypic and immunocytochemistry evaluation for CD44 and vimentin seen in our study were previously described in bovine amniotic fluid-derived MSCs (Rossi et al., 2014). The CD44 and CD29 expression was reported in porcine eMSC (Miernik and Karasinski, 2012), bovine umbilical cord (Xiong et al., 2014), and human eMSCs (Gargett et al., 2009; Verdi et al., 2014). The absence of marking for MHC-II here was maybe due to low immunogenicity of the endometrial cells, which is of great interest for therapeutic use.

Differentiation assays of adipogenic and osteogenic lineages of bovine eMSCs were confirmed in all samples with excellent response after staining (Figure 3). We reinforce the results of Łupicka et al. (2015a,b) which showed that uterine stromal cells differentiated to adipogenic, chondrogenic, and osteogenic lineage upon specific condition, confirming the wide plasticity of these source. We reached great response to differentiation once Donofrio et al. (2008) demonstrated the osteogenic potential of endometrial stromal cells and Cabezas et al. (2014) the osteogenic and chondrogenic potential of endometrial putative mesenchymal progenitor cells from late luteal phase. It could be inferred that when bovine eMSCs are exposed to inducing agents, they have differing sensitivity and hence fail to differentiate in a too early luteal phase.

According to karyotype analysis based on 100% of metaphases, we showed by the first time, until our

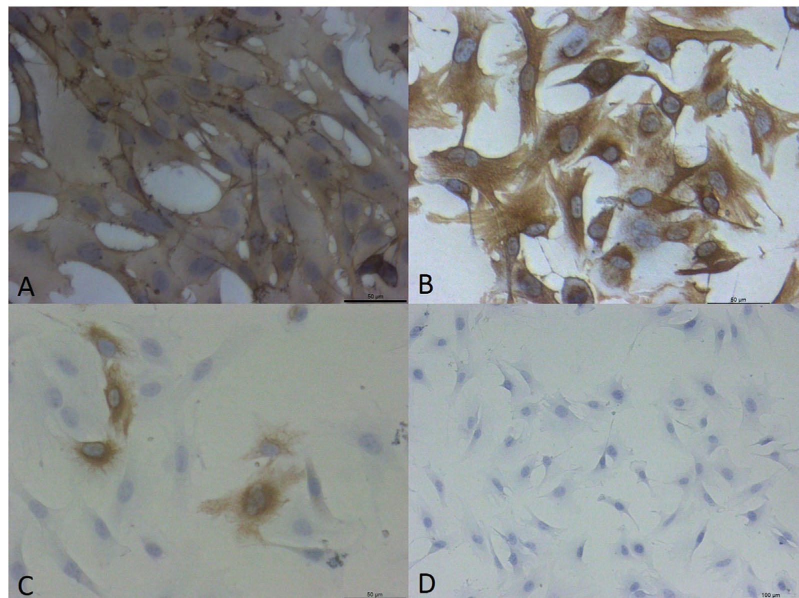


Figure 2 Immunohistochemistry of bovine eMSCs for characterization of the markers CD44 (A), vimentin (B), and cytokeratin (C). The positive staining is shown in brown. Negative control (D) by omission of the primary antibody. Nucleus stained with hematoxylin. Bar = 50 μm (A, B, C), 100 μm (D).

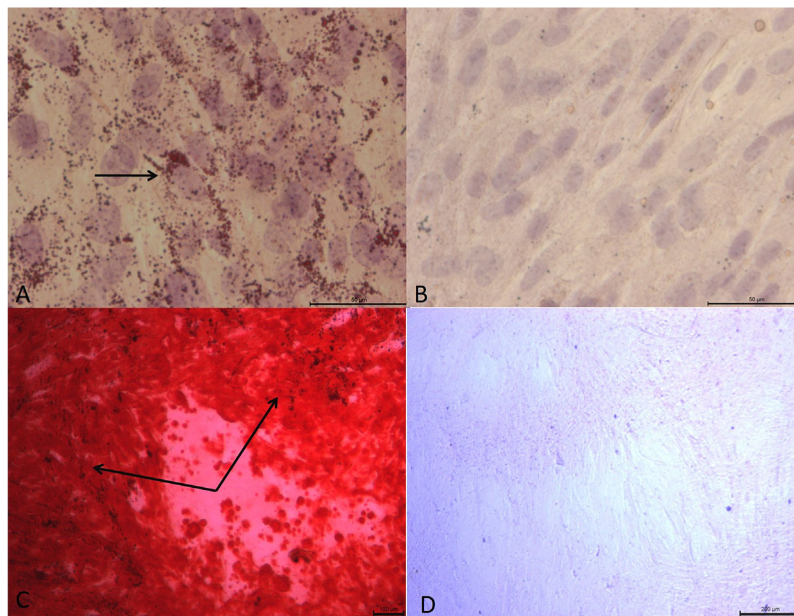


Figure 3 Differentiation assay for adipogenic and osteogenic lineages of bovine eMSCs. Adipogenic differentiation: Note in (A) the presence of intracytoplasmic lipids droplets stained with oil red (arrow) and (B) control of differentiation. Osteogenic differentiation: Note in (C) large calcium deposit stained with Alizarim red (2%) (arrow) and (D) control of differentiation. Bar: 100 μm (A, C) 200 μm (B, D).

knowledge, that these bovine eMSCs have normal chromosome number (2n = 60) (Figure 4B) and chromosomal stability also after cryopreservation (data not shown). This shows that neither isolation technique nor culture or cryopreservation altered the genetic makeup of these cells. Additionally, CE revealed great capacity of clonicity from bovine eMSCs and the formation of delimitated colonies (Figure 4A) without difference ($P = 0.750$) between phases. This CE rate is excellent and comparable to samples from umbilical cords or adipose tissue (Lu et al., 2014; Xiong et al., 2014). Therefore, creating cell biobanks for future use is quite feasible once eMSCs are stable and suitable for therapeutic use.

The cryopreservation using DMSO as a protective solution in combination with a progressive reduction of temperature is efficient in the preservation of good viability rates for bovine cells from amniotic fluid and umbilical cord, once enables the culture after thawed (Cunha et al., 2014). To our knowledge, this is the first report showing the possibility of cryopreservation of bovine eMSCs using two medium of cryopreservation at a controlled temperature rate, allowing the formation of cell biobank. Before cryopreservation cells from phase III presented a better viability rate ($P = 0.01$) and lower proportion of cells on initial apoptosis ($P = 0.04$). A decrease ($P = 0.03$) on the viability rate after cryopreservation was detected on Phase III using both medium, comparing to fresh samples, and at both phases late apoptosis + necrosis increased ($P > 0.05$) on cells cryopreserved with MI and MII comparing to fresh samples. No difference ($P > 0.05$) was detected in any of the variables after cryopreservation comparing the phases

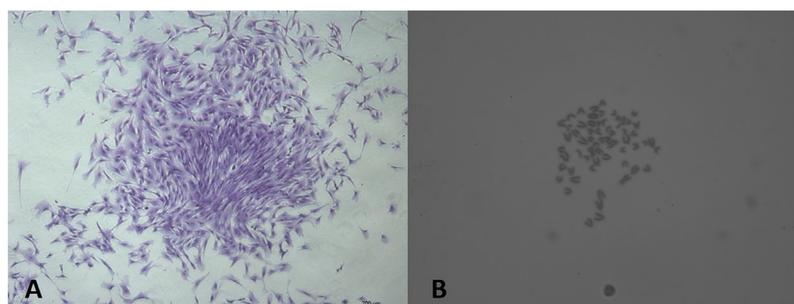


Figure 4 Fibroblastic colony-forming unit assay and karyotype analysis of bovine eMSCs. Note a formation of a colony well defined in (A) and the normal karyotype number of bovine eMSCs (2n = 60) in (B).

Table 1 Data before and after cryopreservation analysis on flow cytometry using anexin V (AN) and propidium iodide (PI) from samples of bovine endometrial mesenchymal stem/progenitor cells from Phases II and III.

	Before cryopreservation		After cryopreservation			
	Phase II	Phase III	Phase II		Phase III	
Variables			Medium 1	Medium 2	Medium 1	Medium 2
Viability (PI− AN−)	85.08 ± 3.88*	93.76 ± 1.09*[a]	81.72 ± 2.32	78.22% ± 2.5	76.08% ± 6.48[b]	67.18% ± 7.39[b]
Necrosis (PI+ AN−)	2.12 ± 0.78	3.00 ± 1.12	3.92% ± 0.79	5.68% ± 2.51	2.6% ± 0.57	1.62% ± 0.15
Late-apoptosis + necrosis (PI+ AN+)	4.54 ± 1.01[a]	1.72 ± 0.58[a]	11.18% ± 2.27[b]	13.28% ± 0.99[b]	13.02% ± 3.36[b]	21% ± 4.77[b]
Initial apoptosis (PI− AN+)	3.78 ± 0.87*	1.48 ± 0.72*	3.14% ± 1.09	2.86% ± 0.67	8.26% ± 3.09	10.14% ± 3.553

Data are presented as mean and SEM.

AN, anexin V; PI, propidium iodide. Different letters between the same phase and medium in the same raw shows statistical difference ($P < 0.05$) before and after cryopreservation. Asterisks represents statistical difference ($P < 0.05$) between phases before cryopreservation.

and medium used. Data before and after cryopreservation are presented at Table 1.

Conclusion

At the present work, we showed for the first time at bovine eMSCs, until our knowledge, the chromosomal stability and potential of cryopreservation with two different mediums. We verified that bovine eMSCs derived from estral uteri adhere to plastic, have fibroblastoid morphology, good clonicity, differentiation potential and immunophenotypic progenitor/stem cells characteristics beside good viability rates after thawing at the two studied phases and with the two medium used. Thus, bovine eMSCs can be used in further studies allowing the formation of biobanks and may have greater potential to impact clinical outcome because they are stable. Depending on the objective (therapeutic use, in vitro studies, formation of cell biobank), cells from both phases could be used, supposing be preferable work with fresh cells from phase III and with cryopreserved cells from phase II according to a possibly better answer and resistance to cryopreservation from this last one. Additionally, both medium used at the present work were suitable to maintain the quality of cells and we highlight the M2 which is commonly discarded and could be used for this propose. Pre-clinical studies of MSCs are still warranted to further establish the most ideal source of MSCs and eMSCs mechanism of action, particularly in potentiating tissue repair.

Acknowledgments and funding

Fundação de Amparo à Pesquisa do Estado de São Paulo for the scholarship (Proc.2014/20447-2, Proc. 2015/18964-1) and financial support (2015/01057-1).

References

Alvarenga MA, Carmo MT, Segabinazzi LG, Guastali MD, Maia L, Landim-Alvarenga FC (2016) Feasibility and safety of endometrial injection of autologous boné marrow mesenchymal stem cells in mare. J Equine Vet Sci 42: 12–8.

Anand V, Dogra N, Singh S, Kumar SN, Jena MK, Malakar D, Dang AK, Mishra BP, Mukhopadhyay TK, Kaushik JK, Mohanty AK (2012) Establishment and characterization of a buffalo (*Bubalus bubalis*) mammary epithelial cell line. PloS ONE 7: 1–14.

Bockeria L, Bogin V, Bockeria O, Alekyan B, Woods EJ, Brown AA, Ichim TE, Patel AN (2013) Endometrial regenerative cells for treatment of heart failure: a new stem cell enters the clinic. J Transl Med 5: 2–8.

Bosnakovski D, Mizuno M, Kim G, Ishiguro T, Okumura M, Iwanaga T, Kadosawa T, Fujinaga T (2004) Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. Exp Hemat 32: 502–9.

Cabezas J, Lara E, Pacha P, Rojas D, Veraguas D, Saravia F, Rodríguez-Alvarez L, Castro FO (2014) The endometium of cycling cows contains populations of putative mesenchymal progenitor cells. Reprod Dom Anim 49: 550–9.

Chan RW, Gargett CE (2006) Identification of label-retaining cells in mouse endometrium. Stem Cells 24: 1529–38.

Chapwanya A, Meade KG, Doherty M, Callanan JJ, ÓFarrelly C (2013) Endometrial epithelial cells are potent producers of tracheal antimicrobial peptide and serum amyloid A3 gene expression in response to *E.coli* stimulation. Vet Immunol Immunopathol 151: 157–62.

Cunha ER, Martins CF, Silva CG, Bessler HC, Báo SN (2014) Effects of prolonged in vitro culture and cryopreservation on viability, DNA fragmentation, chromosome stability and ultrastructure of bovine cells from amminiotic fluid and umbilical cord. Reprod Domes Anim 49: 806–12.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM (2006) Minimal criteria for defining multipotent mesenchymal

stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315–7.

Donofrio G, Franceschi V, Capocefalo A, Cavirani S, Sheldon IM (2008) Bovine endometrial stromal cells display osteogenic properties. Reprod Biol Endocrinol 6: 1–9.

Fortier MA, Guilbault LA, Grasso F (1988) Specific properties of epithelial and stromal cells from the endometrium of cows. J Reprod Fert 83: 239–48.

Gargett CE, Schwab KE, Zilwood RM, Nguyen HPT, Wu D (2009) Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. Biol Reprod 80: 1136–45.

Garget CE, Schwab KE, Deane JA (2015) Endometrial stem/progenitor cells: the first 10 years. Hum Reprod Updade 0: 1–27.

Ireland JJ, Murphee RL, Coulson PB (1980) Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J Dairy Sci 63: 155–60.

Letouzey V, Tan KS, Deane JA, Ulrich D, Gurung S, Ong YR, Gargett CE (2015) Isolation and characterization of mesenchymal stem/stromal cells in the ovine endometrium. PLoS ONE 10: 1–17.

Lu T, Xiong H, Wang K, Wang S, Ma Y, Guan W (2014) Isolation and characterization of adipose-derived mesenchymal stem cells (ADSCs) from cattle. Appl Biochem Biotechnol 174: 717–28.

Łupicka M, Socha B, Szczepańska A, Korzekwa A (2015a) Expression of pluripotency markers in the bovine uterus with adenomyosis. Reprod Biol Endocrinol 29: 1–110.

Łupicka M, Bodek G, Shpigel N, Elnekave E, Korzekwa AJ (2015b) Identification of pluripotent cells in bovine uterus: in situ and in vitro studies. Reproduction 149: 317–27.

Maia L, Landim-Alvarenga FC, Mota LSLS, Golim MA, Laufer-Amorim R, De Vita B, Barberini DJ, Listoni AJ, Moraes CN, Heckler MCT, Amorim RM (2013) Immunophenotypic, immunocytochemistry, ultrastructural and cytogenetic characterizatin of mesenchymal stem cells from equine bone marrow. Micros Res Tech 76: 618–24.

Mambelli LI, Winter GHZ, Kerkis A, Malschutzky E, Mattos RC, Kerkis I (2013) A novel strategy of mesenchymal stem cells delivery in the uterus of mares with endometrosis. Theriogenology 79: 744–50.

Meng X, Ichin TE, Zhong J, Rogers A, Yin Z, Jackson J, Wang H, Ge W, Bogin V, Chan KW, Thébaud B, Riordan NH (2007) Endometrial regenerative cells: a novel stem cells population. J Transl Med 5: 1–10.

Mensing N, Gasse H, Hambruch N, Haeger JD, Pfarrer C, Staszyk C (2011) Isolation and characterization of multipotent mesenchymal stromal cells from the gingiva and the periodontal ligament of the horse. BMC Vet Res 7: 1–13.

Miernik K, Karasinski J (2012) Porcine uterus contains a population of mesenchymal stem cells. Reproduction 143: 203–9.

Ranera B, Ordovás L, Lyahyai J, Bernal ML, Fernades F, Remacha AR, Romero A, Vázquez FJ, Osta R, Cons C, Varona L, Zaragoza P, Martin-Burriel I, Rodellar C (2012) Comparative study of equine bone marrow and adipose tissue derived mesenchymal stromal. Equine Vet J 44: 33–42.

Rossi B, Merlo B, Colleoni S, Iacono E, Tazzari PL, Ricci F, Lazzari G, Galli C (2014) Isolation and in vitro characterization of bovine amniotic fluid derived stem cells at different trimesters of pregnancy. Stem Cell Rev 10: 712–24.

Santamaria X, Massasa EE, Feng Y, Wolff E, Taylor HS (2011) Derivation of insulin producing cells from human endometrial stromal stem cells and use in the treatment of murine diabetes. Mol Ther 19: 2065–71.

Stenger EO, Krishnamurti L, Galipeau J (2015) Mesenchymal stromal cells to modulate immune reconstitution early post-hematopoietic cell transplantation. BMC Immunol 16: 1–10.

Souza D, Rivera L, Quevedo C, Gorino AC, Biagio S, Laufer R (2014) Pulmonary adenocarcinoma in cattle. Revista MVZ Córdoba 19: 4358–63.

Toupadakis CA, Woung A, Genetos DC, Cheung WK, Borjesson DL, Leach JK, Owens SD, Yellowley CE (2010) Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. Am J Vet Res 71: 1237–45.

Verdi J, Tan A, Shoae-Hassani A, Seifalian AM (2014) Endometrial stem cells in regenerative medicine. J Biol Eng 8: 1–10.

Wollf EF, Gao XB, Yao KV, Andrews ZB, Du H, Elsworth JD, Taylor HS (2011) Endometrial stem cell transplantation restores dopamine production in a Parkinson's disease model. J Cell Mol Med 15: 747–55.

Xiong H, Bai C, Wu S, Gao Y, Lu T, Hu Q, Guan W, Ma Y (2014) Biological characterization of mesenchymal stem cells from bovine umbilical cord. Anim Cells Syst 18: 56–67.

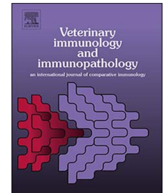
Received 12 May 2016; accepted 30 September 2016.
Final version published online 16 October 2016.

Veterinary Immunology and Immunopathology 187 (2017) 42–47

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



Short communication

Shotgun proteomic analysis of the secretome of bovine endometrial mesenchymal progenitor/stem cells challenged or not with bacterial lipopolysaccharide

Carolina Nogueira de Moraes[a,*], Leandro Maia[a], Eliandre de Oliveira[b], Camila de Paula Freitas Dell'Aqua[a], Aspinas Chapwanya[c], Fernanda da Cruz Landim-Alvarenga[a], Eunice Oba[a]

[a] Department of Animal Reproduction and Radiology, UNESP – São Paulo State University, Botucatu, São Paulo, Brazil
[b] Proteomics Platform, Parc Cientific de Barcelona, Barcelona, Spain
[c] Ross University School of Veterinary Medicine, Department of Clinical Sciences, Basseterre, Saint Kitts and Nevis

ARTICLE INFO

Keywords:
Cow
nanoLC–MS/MS
Protein
Uterus
Endometrial mesenchymal cells

ABSTRACT

The use of the conditioned medium (CM) for diseases treatment is based on its enrichment with biomolecules with therapeutic properties and themselves have a beneficial effect. Secretome of bovine endometrial mesenchymal progenitor/stem cells (eMSCs) using a proteomics approach is until now unknown. This work aimed to evaluate the secretome of bovine eMSCs-CM challenged or not with lipopolysaccharide (LPS). For this, eMSCs characterized were challenged (TG) or not (CG). The CM was collected 12 h after stimulation and submitted to mass spectrometry analysis. The classification of identified proteins was done by PANTHER according to biological processes, molecular function, cellular component and protein class. 397 protein groups were identified in TG and 302 in CG. We observed positive enrichment for antibacterial response proteins, macrophage activation function, receptor-mediated endocytosis, hydrolase activity, inhibitory enzyme in TG, and for activity structural molecule and intermediate filament cytoskeleton in the CG. Our experimental model shows that eMSCs respond to LPS in the concentration used and can be used to study immune-inflammatory response, besides of the secretion of proteins mainly related to tissue remodeling, immune response and angiogenesis which is an interesting feature for use in cell therapy.

1. Introduction

In cattle, the endometrium is the main source of mesenchymal progenitor/stem cells (eMSCs), a small proportion of which are undifferentiated with high plasticity (Lupicka et al., 2015). Bovine eMSCs have been studied because of their biological properties, including the paracrine and immunomodulatory effects, which make them promising for use in cell therapy.

Besides the use of MSCs in therapies, the conditioned medium (CM) has a role in the cellular microenvironment, and can exert a therapeutic effect by accelerating organ regeneration processes (Lavoie and Rosu-Myles, 2013) and tissue repair (Ashiba et al., 2015). MSCs secrete bioactive molecules such as cytokines and growth factors (Ashiba et al., 2015) which are released as soluble molecules or through extracellular vesicles that together are responsible for paracrine (Lavoie and Rosu-Myles, 2013) and autocrine roles related to the regeneration, angiogenesis or modulation of immune responses (Skalnikova, 2013).

MSCs are sensitive to culture media and protein profiles may change in response to microenvironments to which they are subjected. The use of different immunological conditions evaluates the therapeutic potential of MSC-derived molecules and make the study of secreted soluble factors important for the understanding its therapeutic effects (Lavoie and Rosu-Myles, 2013). The use of an experimental *in vitro* model of inflammation using bacterial lipopolysaccharide (LPS) (Lange-Consiglio et al., 2015) makes it possible to evaluate the response of bovine eMSCs facing a stressful insult such as uterine disease.

Interaction of bovine eMSCs-CM is important for understanding how therapeutic approaches can be targeted at the mechanisms by which CM modulates the endometrium or use of eMSCs to treat reproductive pathologies.

The aim of this study was to evaluate the secretome of bovine eMSCs challenged with bacterial LPS by proteomic analysis

* Corresponding author.
E-mail address: carolnmoraes@hotmail.com (C.N. de Moraes).

http://dx.doi.org/10.1016/j.vetimm.2017.03.007
Received 22 June 2016; Received in revised form 6 March 2017; Accepted 23 March 2017
0165-2427/ © 2017 Elsevier B.V. All rights reserved.

(nanoLC–MS/MS) using a shotgun strategy. To the best of our knowledge, this is the first report describing the secretome of bovine eMSCs-CM using proteomic analysis.

2. Material and methods

The study was approved and performed according to the ethical guidelines of the Institution's Animal Care and Experimentation Ethics Committee (Protocol Number 152/2014).

2.1. Isolation and culture of bovine eMSCs

Endometrial cells from bovine endometrial tissue (n = 3) in Phase II of estral cycle (Ireland et al., 1980; Chapawanya et al., 2013) were isolated as previously described (Fortier et al., 1988) with modifications. Briefly, samples were digested with 0.3% trypsin (Sigma ®, USA) in HBSS (Thermo Fisher Scientific®, USA) for 3 h, at 22 °C under agitation. The samples were then filtered with 40 uM filter (Becton Dickinson® and Company, USA). For each sample, a second digestion step was performed with 0.05% mg trypsin, 0.05% collagenase, 0.1% bovine serum albumin and 0.01 mg DNase I (Sigma ®, USA) in HBSS (Thermo Fisher Scientific®, USA) for 1.5 h at 37 °C. After filtration, the digesta was washed with HBSS medium and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific®, USA) twice and centrifuged at 100 x g for 10 min. The sediment was plated and cultured at 37.5 °C in humid atmosphere containing 95% air and 5% CO_2. The culture medium consisted of DMEM high glucose/F12 (1:2), 20% FBS, 100IU/mL penicillin, 100 μg/mL streptomycin, 3 μg/mL amphotericin B (Thermo Fisher Scientific®, USA) and 11 μg/mL amikacin (Teuto®, BRA). The medium was changed within 18 h and every 2–3 days thereafter until the culture reached 90% confluence, and passaged three times.

2.2. Immunophenotypic characterization

The immunophenotypic characterization (n = 2) was performed by flow cytometry at a LSR Fortessa equipment (BD®, BR) using the antibodies anti–CD29 conjugated with Alexa fluor 647 (TS2/16, BioLegend®, USA), mouse anti-bovine CD-44 conjugated with fluorescein isothiocyanate (FITC) (IL-A118, AbD Serotec®, UK), mouse anti-horse MHC–II conjugated with FITC (CVS20, AbD Serotec®, UK), rabbit anti CD-34 conjugated with FITC (polyclonal, Biorbyt®, USA) and mouse anti-vimentin (v9, AbD Serotec®, UK). A secondary goat-anti mouse conjugated with FITC (abcam®, USA) was used for mouse anti-vimentin. All these antibodies cross-react with bovine antigens (Moraes et al., 2016). Fluorescence reactions were analyzed using the BD FACSDiva™ software and were accounted for 10,000 events. The debris population were excluded by gating FSC x SSC at FSC 5000 threshold. Markers with expression levels of ≥ 2% were considered positive. Data from immunophenotypic characterization is presented as mean and standard error of the mean.

2.3. Immunocytochemistry characterization

Immunocytochemistry (n = 3) was performed as previously described (Maia et al., 2013) and the reactions evaluated under an inverted light microscope (Leica® Microsystems, GER) using the software Leica Application Suite (LAS), version 4.3.0. The antibodies evaluated were vimentin (1: 200, V9, AbD Serotec®, UK), pan-cytokeratin (1: 100, C11, abcam®, USA) and CD-44 (1: 100, BAG40A, VMRD®, USA).

2.4. Assays for differentiation

After attaining 95% confluence, assays for differentiation of adipogenic and osteogenic lineages (n = 3) were performed by adding media (StemPro, Thermo Fisher Scientific®, USA) to the subcultures in triplicate, and also supplementing 5% rabbit serum (Maia et al., 2013) or 20% FBS.

Osteogenic differentiation was confirmed on the 14th day when calcium matrix deposits were noted on Alizarin red stains (Sigma®, USA). Confirmation of adipogenic differentiation on the 8th day was by presence of intracytoplasmic fat droplets after staining with 0.5% Oil red (Sigma®, USA).

2.5. Challenge of bovine eMSCs with LPS

For evaluating the protein profile of the secretome, bovine eMSCs were plated on 24 wells (2 cm^2) at a density of 1000 cells/cm^2 and cultured with complete maintenance medium (DMEM high glucose/F12 (1:2), 20% FBS, 100IU/mL penicillin, 100 μg/mL streptomycin, 3 μg/mL amphotericin B; Thermo Fisher Scientific®, (USA), 11 μg/mL amikacin Teuto®, (BRA)). After 60–70% confluence, the cells were cultured in maintenance medium without FBS for 24 h. The control (CG; n = 3) and LPS-stimulated (LPS treated, TG; n = 3) were cultured.

After 12 h, the conditioned medium was collected, filtered through 22 μm filter and centrifuged at 2000g for 5 min to remove cellular debris, and the supernatant stored at −86 °C for secretome analysis.

2.6. Secretome analysis by mass spectrometry and liquid chromatography (nanoLC–MS/MS)

Three biological replicates in both groups (treated vs. control) were analyzed. The samples were digested by initially denaturing in 8 M urea solution (Sigma 51459), followed by reduction with 50 mM dithiothreitol (32 °C/60 min, Sigma 9779). An alkylation step was then performed with 150 mM iodocetamida (25 °C/30 min in the dark) followed by digestion with trypsin sequence grade (35 °C, 16 h, Promega V511A). After digestion, the samples were clean-up with C18 reverse phase and strong cationic-exchange columns (C18, SCX, PolyLC). The samples were then analyzed in a nanoAcquity liquid chromatographer (Waters) coupled to a LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer.

2.7. Data analysis

Thermo Proteome Discover (v.1.4.1.14) was used to search with SequestHT search engine against Mammalia-SwissProt + Bos taurus-TREMBL protein database (v. april 2016). The search parameters used were: Enzyme: Trypsin; Missed Cleavage: 2; Precursor and Fragment Mass Tolerances: 10 ppm and 0.6 Da, respectively; Variable and Static: Oxidation methionine and Carbamidomethyl cysteine, respectively.

Gene ontology protein classification analysis according biological process (BP), molecular function (MF), cellular component (CC) and protein class (PC) was performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (http://pantherdb.org/).

3. Results and discussion

Bovine eMSCs isolated from uteri in Phase II of the estral cycle were cultured and characterized by immunophenotyping and immunocytochemistry. The cells were also assessed for differentiation potential in addition to being challenged with LPS for secretome analysis using a proteomic approach.

The eMSCs adhered to plastic surfaces within six hours of culture, and showed fibroblastoid morphology after passaging (Fig. 1). Immunophenotypic evaluation by FC revealed high expression for the markers vimentin (94.35% ± 2.19), CD-29 (99.85% ± 0.07) and CD-44 (96.9% ± 2.40). Similar to previous studies (Xiong et al., 2014), there was low expression of the CD-34 (4.25% ± 1.06) marker, and no expression of the MHC-II marker (1.05% ± 0.78) (Fig. 2). After evaluating for osteogenic and adipogenic potential, the eMSCs cells

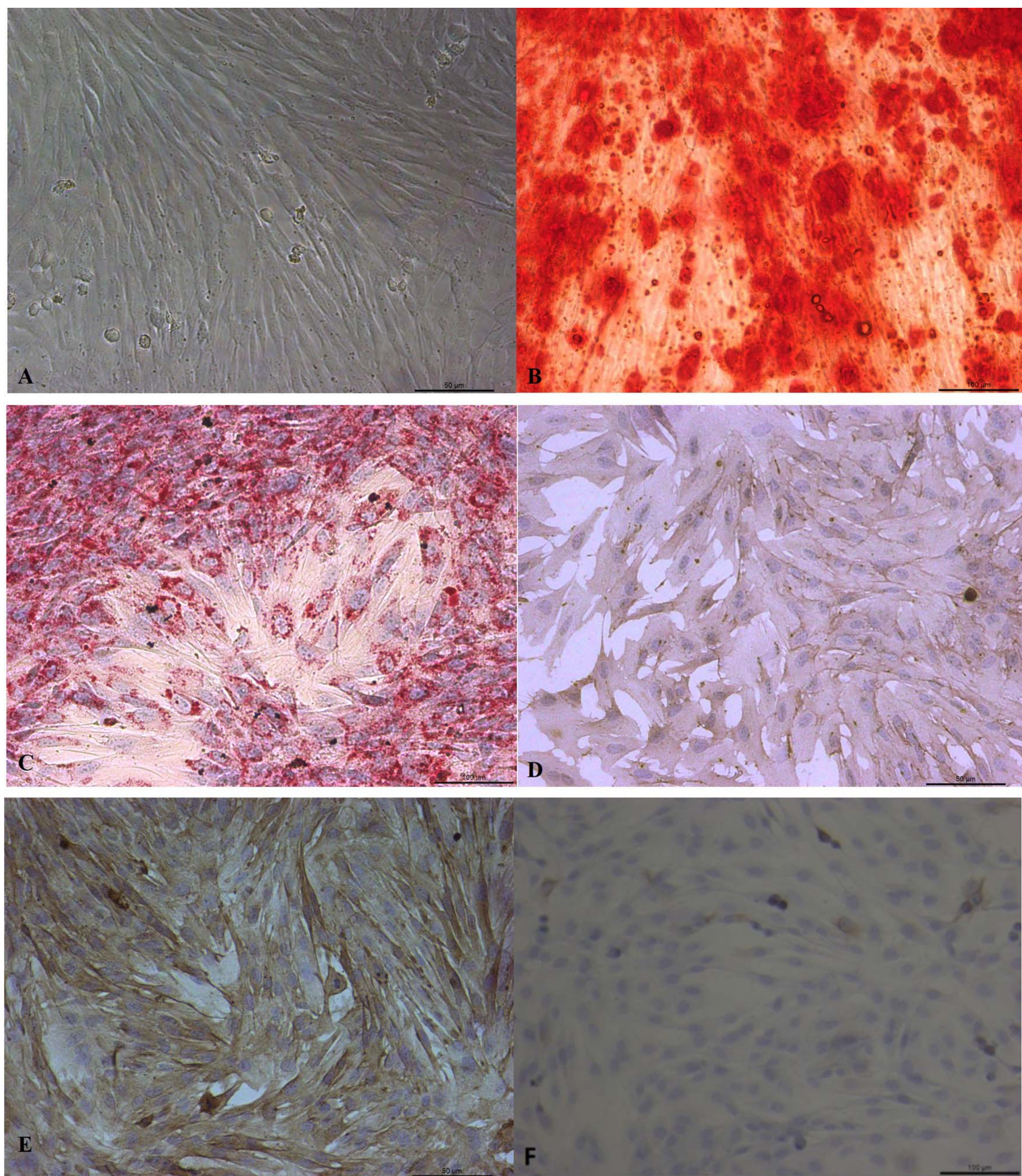


Fig. 1. Characterization of bovine eMSCs. A—Bovine eMSCs after first passage adhere to plastic surfaces and show fibrobastoid morphology. B—eMSCs differentiate into osteogenic lineage after the third passage. Calcium deposits stained with Alizarim are shown. C—eMSCs differentiate into adipogenic lineage of bovine eMSCs in third passage. Intracytoplasmic lipids droplets stained with Oil red are shown. Immunohistochemical staining of bovine eMSCs for CD-44 (D), Vimentin (E) and Pancytokeratin (F). Brown staining indicates positive. Hematoxylin stained cell nuclei. Size: 100 µm (A, D, F), 200 µm (B, C, E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stained positive, similar to previous finding (Lupicka et al., 2015). Similarly, other authors reported positive staining for the markers CD-44 in bovine endometrial putative mesenchymal cells on immunocytochemistry (Cabezas et al., 2014), and for vimentin in human eMSCs (Kato, 2012) (Fig. 1). Here, some endothelial epithelial cells were positive for pan cytokeratin, which showed that there were no cell morphology alterations after culture.

A proteomic analysis was performed in order to find more information on the protein content of the secretome of bovine eMSCs. A shotgun approach was used and the LC–MS/MS detected a total of 397 proteins groups on TG and 302 on CG with 242 commons between the groups (Fig. 3). Many proteins belonging to the immune system, angiogenic processes, antioxidant and tissue remodeling pathways were detected in the TG (Table 1).

Our *in vitro* experimental model showed that the cells respond positively and in a protective manner after stimulation with LPS. It can be inferred, for instance, from the presence of arginase I and heat shock proteins, that these proteins protect cells or tissues from stress (Frier and Locke, 2007) by stabilizing and repairing proteins (Fan, 2012). This response is characteristic of a defense mechanism via the secretion of crucial proteins for tissue restoration, or protection from tissue injury. This suggests that CM alone or together with MSCs can be an efficient alternative to assist in healing (Ashiba et al., 2015).

MSCs are sensitive to culture media and may change their proteomic profiles in response to the microenvironments in which they are subjected (Lavoie & -Rosu Myles, 2013). It is noteworthy that in the CM of CG, there were proteins with antimicrobial or antifungal activity, and also tissue remodeling (Table 1). The presence of these proteins in

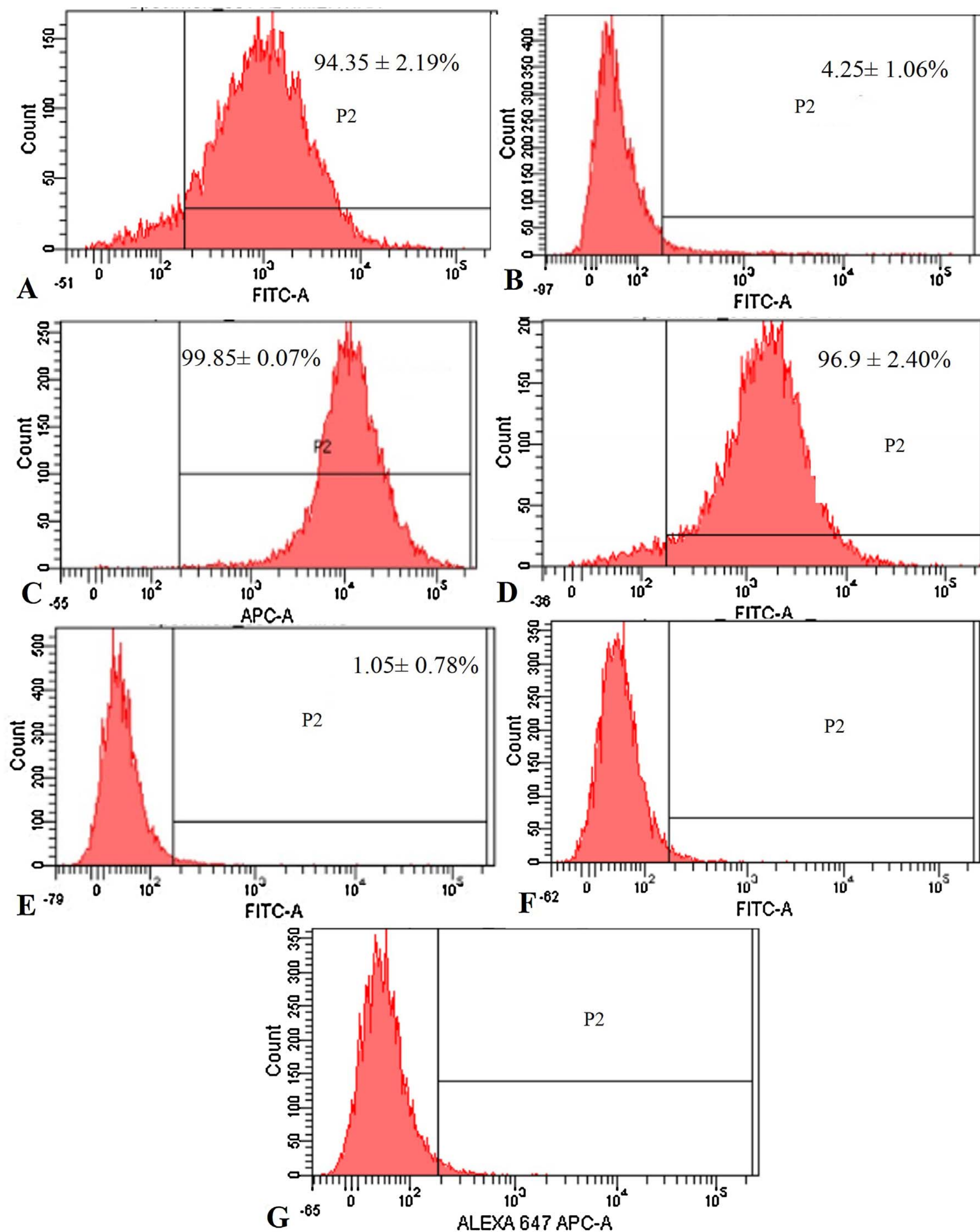


Fig. 2. Histograms of the bovine eMSCs cytomarkers analyzed by flow cytometry. A − Vimentin; B − CD34; C − CD29; D − CD44; E − MHC-II; F and G: controls.

the CM of CG, even without stimulation, could be promising to demonstrate the great potential and possibility of using this medium for use as therapeutics. In humans, the CM of MSCs from dental pulp was efficient in the experimental treatment of multiple sclerosis and even showed similar effects of treatment with their own MSCs (Shimojima et al., 2016).

A protein with antioxidant activity (peroxiredoxin-6, Table 1) was found in TG. This antioxidant capacity of the CM was previously reported for restoring or reducing retinal functions in diabetes animal model (Duarte et al., 2016).

Recently, Lange-Consiglio et al. (2015) showed in an experimental equine uterine inflammation model that horse amniotic MSCs-CM significantly reduce the expression of MMP-1 and MMP-13. These genes modulate inflammatory pathways. Also, in humans, CM of uterine cervical stem cells showed to have anti-inflammatory and bactericidal roles (Bermudez et al., 2015) and the eMSCs showed a potential to be used at pelvic organ prolapse once cells can induce the immune response and help at the tissue reorganization (Emmerson and Gargett, 2016). In our study, anti-inflammatory proteins (such as granulins, Table 1) were found in TG. In addition, *in vitro* use of the CM associated with endometrial cells resulted in improvement of the proliferation rate, showing the relevance of soluble factors produced by MSCs and its potential to increase cell replacement (Corradetti et al., 2014). Such studies support using CM as an alternative for treating

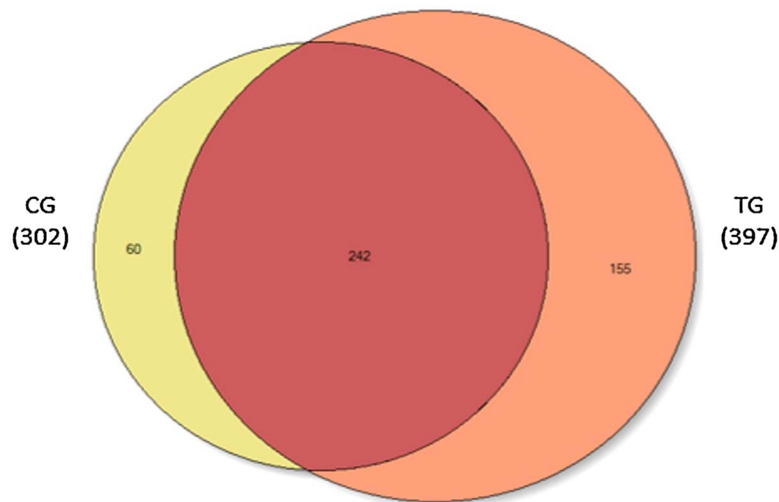


Fig. 3. Venn diagram of the proteins identified in the present study with at least one peptide sequence in the treated (TG) and control (CG) groups. The False discovery rate was ≤1%.

uterine diseases causing low fertility in cattle.

To better characterize the CM proteome, we performed some classification on the identified proteins using Gene Ontology tools (http://geneontology.org/). The Panther GO analysis identified positive enrichment (PE) for BP in macrophage activation function (GO: 0042116, fold enrichment, FE = 7.87) and receptor-mediated endocytosis (GO: 0006898, FE = 5.23) in TG. Regarding MF, there was PE for hydrolase activity (GO: 0016787; FE = 1.87) and inhibitory enzyme (GO: 0004857; FE = 5.15) in the TG, and activity structural molecule (GO: 0005198; FE = 2.73) in the CG. For CC, enrichments were verified

Table 1
Proteins with more than two PCMs differently expressed in the treated (TG) and control (CG) groups. These proteins have mainly tissue repair and immunomodulatory roles.

Biological Functions	Swiss-Prot accession	Protein name	Gene name	Group
Immune response	Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1	Control
	KRT16	Uncharacterized protein	GP340	Control
	P01857	Ig gamma-1 chain C region	KRT16	Control
	A5A6M2	Annexin A1	IGHG1	Control
	P01876	Ig alpha-1 chain C region	ANXA1	Control
	P01834	Ig kappa chain C region	IGHA1	Control
			IGKC	
Antimicrobian/antifungal activity	P03973	Antileukoproteinase	SLPI	Control
	P59665	Neutrophil defensin 1	DEFA1	Control
	P06702	Protein S100-A9	S100A9	Control
	P01857	Ig gamma-1 chain C region	IGHG1	Control
	Q3T0Z0	Uncharacterized protein (WAP four-disulfide core domain 2)	WFDC2	Control
	P81644	Apolipoprotein A-II	APOA2	Treated
	G3N3P6	Cystatin	N/A	Treated
	F1MI18	Uncharacterized protein	N/A	Treated
	F1MNV5	Kininogen-1	N/A	Treated
	F1MVS9	Uncharacterized protein	KNG1	Treated
	P31944	Caspase 14	MASP1	Treated
	A2I7M9	Serpin A3-7	CASP14	Treated
	Q1JPB0	Leukocyte elastase inhibitor (LEI)	SERPINA3-2	Treated
			SERPINB1	
Anti-inflammatory activity	A5A6M2	Annexin A1	ANXA1	Control
	P06702	Protein S100-A9	S100A9	Control
	P28799	Granulins	GRN	Treated
Tissue remodeling	A5A6M2	Annexin A1	ANXA1	Control
	E2DI12	Syndecan-1 (Fragment)	SDC1	Control
	P28799	Granulins	GRN	Treated
	P50757	72 kDa type IV collagenase	MMP2	Treated
	E1B726	Plasminogen	PLG	Treated
	F1N2Y2	Uncharacterized protein	COL5A2	Treated
	E1B726	Plasminogen	PLG	Treated
Angiogenic activity	P80929	Angiogenin-2	ANG2	Treated
	Q2KIF2	Leucine-rich alpha-2-glycoprotein 1	LRG1	Treated
	P50757	72 kDa type IV collagenase	MMP2	Treated
Protective activity	E9RHW1	Heat shock 27 kDa protein 1	HSPB1	Treated
Antioxidant	P86215	Peroxiredoxin-6	PRDX6	Treated
Other functions	A0SXL6	Elongation factor 2 (EF-2)	EEF2	Control
Cell/cell adhesion	A7YWB6	Transforming growth factor-beta-induced protein ig-h3	TGFBI	Treated
Adhesion/cell aggregation	A5PJE3	Fibrinogen alpha chain	FGA	Treated
Platelet aggregation	P05997	Collagen alpha-2(V) chain	COL5A2	Treated
Extracellular matrix constituent	Q61245	Collagen alpha-1(XI) chain	COL11a1	Treated

in both groups to extracellular matrix (GO: 0031012) and extracellular region (GO: 0005576), as well as intermediate filament cytoskeleton (GO: 0045111) in the CG. Interestingly, with respect to PC we evidenced PE in antibacterial response proteins (PC00051; FE = 5.87) in the TG.

Here we observed proteins with anti-inflammatory, antibacterial properties and related to tissue remodeling in both CM which leads us to believe that these cells respond to many stimuli to defend the organisms. Thus, eMSCs and CM may have a role in treating reproductive tract diseases in cattle. Also, our results, especially based on protein enrichment to macrophage activation and identification of large number of proteins related to immune response on TG, allow us to infer that our *in vitro* model of stimulation with bacterial LPS of bovine eMSCs is effective to study immune and inflammatory response.

Acknowledgements

The authors thank the Proteomics Platform of the Barcelona Science Park. We are also indebted to PRB2, ProteoRed-ISCIII, the Spanish Proteomics network. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo [Proc.2014/20447-2, Proc. 2015/18964-1, Proc.2015/01057-1].

References

Ashiba, K., Terunuma, A., Terunima, H., Takane, T., Deng, X., Yamashita, Y., Watanabe, K., 2015. Immortalized mesenchymal stem cells producing conditioned medium in a large scale for −t, herapeutic useage. Inflamm. Regen. 35, 57–60. http://dx.doi.org/10.2492/inflammregen.35.057.

Bermudez, M.A., Sendon-Lago, J., Eiro, N., Trevino, M., Gonzales, F., Yebra-Pimentel, E., Giraldez, M.J., Macia, M., Lamelas, M.L., Saa, J., Vizoso, F., Perez-Fernandez, R., 2015. Corneal epithelial wound healing and bactericidal effect of conditioned medium from human uterine cervical stem cells. Cornea 56 (2), 983–992. http://dx.doi.org/10.1167/iovs.14-15859.

Cabezas, J., Lara, E., Pacha, P., Rojas, D., Veraguas, D., Saravia, F., Rodríguez-Alvarez, L., Castro, F.O., 2014. The endometium of cycling cows contains populations of putative mesenchymal progenitor cells. Reprod. Dom. Anim. 49, 550–559. http://dx.doi.org/10.1111/rda.12309.

Chapawanya, A., Meade, K.G., Doherty, M.L., Callanan, J.J., O'Farrelly, C., 2013. Endometrial epithelial cells are potent producers of tracheal antimicrobial peptide and serum amyloid A3 gene expression in response to *E. coli* stimulation. Vet. Immunol. Immunopathol. 151, 157–162. http://dx.doi.org/10.1016/j.vetimm.2012.09.042.

Corradetti, B., Correani, A., Romaldini, A., Marini, M.G., Bizarro, D., Perrini, C., Cremonesi, F., Lange-Consiglio, A., 2014. Amniotic membrane-derived mesenchymal cells and their conditioned media: potential candidates for uterine regenerative therapy in the horse. PLoS One 9, 1–9. http://dx.doi.org/10.1371/journal.pone.0111324.

Duarte, D.A., Papadimitriou, A., Gilbert, R.E., Thai, K., Zhang, Y., Rosales, M.A.B., Faria, J.B.L., Faria, J.M.L., 2016. Conditioned medium from early- outgrowth bone marrow cells is retinal protective in experimental model of diabetes. PLoS One 11, 1–15. http://dx.doi.org/10.1371/journal.pone.0147978.

Emmerson, S.J., Gargett, C.E., 2016. Endometrial mesenchymal stem cells as a cell based therapy for pelvic organ prolapse. World J. Stem Cell 8, 202–215. http://dx.doi.org/10.4252/WJSC.v8.i5.202.

Fan, G.C., 2012. Role of heat shock proteins in stem cell behavior. Prog. Mol. Biol. Transl. Sci. 11, 305–322. http://dx.doi.org/10.1016/B978-0-12-398459-3.00014-9.

Fortier, M.A., Guilbault, L.A., Grasso, F., 1988. Specific properties of epithelial and stromal cells from the endometrium of cows. J. Reprod. Fert. 83, 239–248.

Frier, B.C., Locke, M., 2007. Heat stress inhibits skeletal muscle hypertrophy. Cell Stress Chapereones 12, 132–141. http://dx.doi.org/10.1379/CSC-233R.1.

Kato, K., 2012. Stem cells in human normal endometrium and endometrial cancer cells: characterization of side population cells. Kaohsiung J. Med. Sci. 28, 63–71. http://dx.doi.org/10.1016/j.kjms.2011.06.028.

Lange-Consiglio, A., Perrini, C., Esposti, P., Derigibus, M.C., Camussi, G., Pascucci, L., Marini, M.G., Corradetti, B., Bizarro, D., Cremonesi, F., 2015. Effects of microvesicles secreted from equine amniotic-derived progenitor cells on in vitro lipopolysaccharide-treated tendon and endometrial cells. Reprod. Fertil. Dev. 28, 244–245. http://dx.doi.org/10.1071/RDv28n2Ab226.

Lavoie, J.R., Rosu-Myles, M., 2013. Uncovering the secretes of mesenchymal stem cells. Biochimie 95, 2212–2221. http://dx.doi.org/10.1016/j.biochi.2013.06.017.

Lupicka, M., Bodek, G., Shpigel, N., Elnekave, E., Korzekwa, A.J., 2015. Identification of pluripotent cells in bovine uterus: in situ and in vitro studies. Reproduction 149, 317–327. http://dx.doi.org/10.1530/REP-14-0348.

Maia, L., Landim-Alvarenga, F.C., Mota, L.S.L.S., Golim, M.A., Laufer-Amorim, R., De Vita, B., Barberini, D.J., Listoni, A.J., Moraes, C.N., Heckler, M.C.T., Amorim, R.M., 2013. Immunophenotypic, immunocytochemistry, ultrastructural and cytogenetic characterization of mesenchymal stem cells from equine bone marrow. Micros Res. Tech. 76, 618–624. http://dx.doi.org/10.1002/jemt.22208.

Moraes, C.N., Maia, L., Camargos, M.D., Freitas-Dell' Aqua, C.P., Mota, L.S.L.S., Chapwanya, A., Landim-Alvarenga, F.C., Oba, E., 2016. Bovine endometrial cells: a promising source of mesenchymal stem/progenitor cells. Cell Biol. Int. 40, 1332–1339. http://dx.doi.org/10.1002/cbin.10688.

Shimojima, C., Takeuchi, H., Jin, S., Parajuli, B., Hattori, H., Suzumura, A., Hibi, H., Ueda, M., Yamamoto, A., 2016. Conditioned medium from the stem cells of human exfoliated deciduous teeth ameliorates experimental autoimune encephalomyelitis. J. Immunol. 15, 4164–4171. http://dx.doi.org/10.4049/jimmunol.1501457.

Skalnikova, H.K., 2013. Proteomic techniques for characterization of mesenchymal stem cell secretome. Biochimie 95, 2196–2211. http://dx.doi.org/10.1016/j.biochi.2013.07.015.

Xiong, H., Bai, C., Wu, S., Gao, Y., Lu, T., Hu, Q., Guan, W., Ma, Y., 2014. Biological characterization of mesenchymal stem cells from bovine umbilical cord. Anim. Cells Syst. 18, 56–67. http://dx.doi.org/10.1080/19768354.2014.880370.