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Short communication

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

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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

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(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 2000*g* 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