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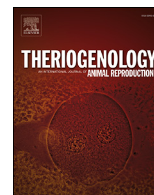

A proteomic study of mesenchymal stem cells from equine umbilical cord

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To the best of our knowledge, this is the first study describing the proteome of equine umbilical cord intervascular matrix mesenchymal stem cells (UCIM-MSCs) in a global and functional manner. The aim of this work was to analyze the proteome of previously characterized UCIM-MSCs to determine protein abundance and classify the identified proteins according to Gene Ontology (GO) terms. Protein classification analysis according to biological process, molecular function and cellular component was performed using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System, which revealed enrichment for 42 biological processes, 23 molecular functions and 18 cellular components. Protein abundance was estimated according to the emPAI method (Exponential Modified Protein Abundance Index). The two most abundant proteins in the proteome of UCIM-MSCs were the cytoskeletal proteins actin and vimentin, which have important roles in cell stability and motility. Additionally, we identified 14 cell surface antigens. Three of them, CD44, CD90 and CD105, had been previously validated by flow cytometry. In the present study, we also identified important information about the biological properties of UCIM-MSCs such as differentiation potential, low immunogenicity (low MHC-II expression) and chromosomal stability, which reinforces their use for cell therapy. Together with the proteomic findings, this information allowed us to infer the functional relevance of several activities related to primary metabolic processes, protein synthesis, production of vesicle coats, vesicle-mediated transport and antioxidant activity. In addition, the identification of different cell surface markers may help establish an immunophenotypic panel suitable for the characterization of MSCs from equine fetal membranes.

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1. Introduction

Mesenchymal stem cells (MSCs) have emerged as promising candidates for cell therapy given their numerous biological proprieties and benefits. The safety and feasibility of MSC transplantation [1], as well as satisfactory therapeutic responses, have been demonstrated in the literature for equine species [2,3].

In equine medicine, MSCs from bone marrow (BM-MSCs) and adipose tissue (AT) are considered the two most common types of adult stem cells (ASC) used in tissue regeneration [4] and *in vitro* studies. However, other sources of SCs such as fetal membranes (FM), including the umbilical cord (UC), have attracted great interest in the scientific community because their differentiation potency lies between that of embryonic stem cells and multipotent ASCs. Moreover, FM-derived SCs are also more ethically acceptable in human medicine and are thus attractive for regenerative

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therapies and biotechnological applications [5].

Proteins are key cellular components and have several features that cannot be predicted from their genetic sequences or transcription levels. For example, post-translational modifications, protein-protein interactions, and subcellular location can all affect protein function and activity [6]. Proteins play broad and complex roles in biological processes, and every step of cell generation, from DNA replication to senescence and death, depends on correct protein function [7]. Proteomics can be defined as the direct qualitative and quantitative analysis of all the proteins in an organism, tissue or cell under a given set of environmental and physiological conditions [8].

Many of the open questions in basic and clinical SC research can be answered most efficiently with proteomic analysis [6]. For example, proteomics can be used to study global protein profiles, the processes of differentiation and maturation, and the production of paracrine and autocrine factors [9]. In humans, the identification of protein markers may be useful for characterization, monitoring the differentiation and proliferation of MSCs, thus contributing to the discovery of therapeutic targets [10]. Likewise, such information when obtained through proteomic studies may contribute to the clinical application of MSCs in veterinary medicine.

Proteomic studies in equine medicine are still lacking, especially in the SC field. To the best of our knowledge, this is the first study to describe the proteome of equine umbilical cord mesenchymal stem cells in a global and functional manner by Gene Ontology (GO) classification. The aim of this work was to perform shotgun proteomic analysis on protein extracts from previously characterized mesenchymal stem cells from the intervascular matrix of equine umbilical cord and to classify the identified proteins according to GO terms. In addition, a semi-quantitative (Exponential Modified Protein Abundance Index) emPAI [11] analysis was performed to estimate the concentrations of the most abundant and particularly interesting proteins in the MSC proteome. These results will contribute to a better understanding of the biological properties of MSCs from fetal membranes. Additionally, proteins identified in the proteome of UCIM-MSCs, as well as their functional analysis are of great value for the directing of the cellular therapy in function of the clinical disorder and affected tissue. They will also lead to the identification of new biomarkers, thereby improving the characterization and therapeutic applications of equine MSCs.

2. Materials and methods

The experimental protocol (34/2013-CEUA) was approved by the ethics and welfare committee of Sao Paulo State University - Botucatu.

2.1. Collection of umbilical cord

Equine UC samples (N = 6) were collected at a thoroughbred farm. A segment of UC approximately 10 cm in length was collected and washed with phosphate-buffered saline (PBS), pH 7.4, containing antibiotics (1%) and an antimycotic (1.2%). Then, the samples were transferred into a 50-mL tube containing medium composed of DMEM (Dulbecco's Modified Eagle's Medium) high glucose (4.5 g/L), 20% fetal bovine serum (FBS), penicillin (100IU/mL), streptomycin (100 mg/mL) and amphotericin (3 μg/mL) (Thermo Fisher Scientific®, USA) and were stored and transported at 5 °C in a refrigerated transport system (Botutainer®, Botupharma, BRA).

2.2. Isolation and culture of UCIM-MSCs

The UC samples were first immersed in 70% ethanol [12,13] and then washed in HBSS with penicillin/streptomycin (1%), amphotericin B (1.2%) (Thermo Fisher Scientific®, USA) and amikacin (22 μg/mL). Subsequently, the UCs were dissected for complete removal of blood vessels, and the intervascular portion of the matrix was separated, fragmented, and digested with 1 mg/mL collagenase (Sigma-Aldrich®, USA) at 37 °C. After enzymatic digestion, the samples were filtered through a 70-μm filter (Becton Dickinson and Company®, USA) and subsequently centrifuged with DMEM low glucose (1 g/L) at a ratio of 1:1 at 350 g for 10 min. Then, the supernatant was discarded and the pellet was resuspended and cultured in DMEM high glucose (4.5 g/L), 20% fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (3 μg/mL) (Thermo Fisher Scientific®, USA) and amikacin (11 μg/mL) (Teuto®, BRA) at 37.5 °C in a humidified atmosphere containing 95% air and 5% CO_2. The maintenance medium was changed every 2 or 3 days until cell confluency reached at least 80%, at which point cells were trypsinized to obtain subcultures for characterization and protein extraction.

2.3. Assay for cellular differentiation into mesodermal lineages

Second passage (2P) UCIM-MSCs (N = 6) were seeded in six-well plates (Sarstedt®, USA) with maintenance medium. Forty-eight hours after incubation, the medium was removed and replaced with STEMPRO® osteogenic or adipogenic differentiation medium (Thermo Fisher Scientific®, USA) according to the manufacturer's recommendations. Adipogenic and osteogenic media were supplemented with 5% rabbit serum [14] and 20% FBS [15], respectively.

Differentiation medium was changed every two to three days and confirmation of osteogenic and adipogenic differentiation were performed, respectively, by the observation of calcified extracellular matrix deposits using 2% Alizarin red staining, pH 4.2 (Sigma-Aldrich®, USA), and the presence of intracytoplasmic lipids droplets using 0.5% Oil Red O (Sigma-Aldrich®, USA). We note that for one sample, cell detachment prevented the evaluation of adipogenic differentiation.

2.4. Flow cytometry analysis

Immunophenotypic analysis was performed in second passage (2P) UCIM-MSCs (N = 6) according to the methods described by Maia et al. [14] with modifications. Characterization was performed using a LSRFORTESSA flow cytometer (Becton Dickinson and Company®, USA) with monoclonal antibodies anti-horseCD44 (clone CVS18, AbD Serotec®, USA), anti-horse MHC class II (CVS20, AbD Serotec®, USA), anti-rat CD90 (clone OX7, AbD Serotec®, USA), anti-human CD34 (clone 581/CD34, Becton Dickinson and Company®, USA) and anti-human CD105 (clone SN6, AbD Serotec®, USA) antibodies labeled with fluorescein isothiocyanate. During the analyses, 10.000 events were recorded. The results were presented as the mean and standard error of the mean.

2.5. Colony-forming unit fibroblastic assay (CFU-F)

The self-renewal capacity and efficiency of UCIM-MSCs (N = 6) was evaluated using the CFU-F assay, in triplicate, according to the methodology described by Mensing et al. [16] with modifications (seeding density and culture period were determined based on preliminary pilot studies). The UCIM-MSCSs (2P) were plated at a low density (2000 cells/well) in six-well plates. On day 5, the cultures were fixed and stained with 1% violet crystal in methanol. Stained colonies with more than 20 cells were classified as CFU-F and counted. The colony-forming efficiency was calculated using the following formula: CFU-F = efficiency (CFU-F counted/seeding

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