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HUMAN LACTOFERRIN CAN ENHANCE THE OSTEOGENIC DIFFERENTIATION OF EQUINE MESENCHYMAL STEM CELLS?

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Abstract

Mesenchymal stem cells (MSCs) are harvested after birth; they are adult stem cells and, due to their unique potential, are considered very valuable tools for equine regenerative medicine. MSCs have self-renewal capacity and multilinear differentiation potential. Multiple protocols are used to induce the directed differentiation of these cells. The aim of our study was to evaluate the osteoinductive potential of a glycoprotein from the transferrin family, lactoferrin (Lf) on MSCs isolated from equine synovial fluid. The cell line (syMSCs) used for this study was obtained from synovial fluid samples from a healthy horse. The isolated cells were characterized morphologically, immunophenotypically and functionally respecting the standards of the International Society for Cell Therapy which were originally drawn up for human MSCs (cellular plastic adherence, expression of specific surface markers and trilinear differentiation capacity). The cells were cultivated in normal propagation medium for MSCs. For osteogenic differentiation, syMSCs were seeded at a concentration of 1 × 10^5 cells/3 mm well, and cultured in osteogenic induction medium with (3 different concentration: 20, 50, 100 µg/mL) and without Lf. The proliferation potential of the cells were assessed using CCK8 assay and the markers of osteogenic differentiation (alkaline phosphatase, ALP) were detected using fluorimetric assay. Our results demonstrate the osteogenic potentiation capacity of human lactoferrin correlated with concentration, thus our future studies will try to elucidate the osteoinductive mechanism of lactoferrin by applying genomics and proteomics techniques.

Key words*: mesenchymal stem cells, equine, differentiation, osteogenic, lactoferrin.*

INTRODUCTION

Mesenchymal stem cells (MSCs) are cells of mesodermal origin; they are multipotent adult cells with important biological properties for regenerative medicine (Cequier et al., 2021). These cells can be harvested from different tissues, are self-renewal capacity and multilinear differentiation potential (Pall et al., 2016). Equine mesenchymal stem cells have been isolated from multiple tissues, namely bone marrow, peripheral blood, umbilical cord blood, adipose tissue, placenta, (Prado et al., 2015), synovial membrane (Sakaguchi et al., 2005) and synovial fluid (Crecan et al., 2019). MSCs have fusiform morphology, with adhesion capacity and high proliferation potential. According to the International Society of Cellular Therapy, there is a set of standards to define human MSCs: adhesion on the cultivation surface, the expression of specific surface molecules (CD105, CD73, CD90, lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR) and trilinear differentiation capacity (Dominici et al., 2006). *In vivo* MSCs have the ability to differentiate in addition to the chondrogenic and adipogenic lineages to the osteogenic lineges. Osteogenic differentiation can be induced *in vitro* using specific osteogenic induction medium. Different substances such as multi-bioactive mesoporous silica nanoparticles, curcumin, quercetin, kaempferol, naringin and other flavonoids, can also induce this ability (Tavares et al., 2020, Xiong et al., 2020; Zhang et al., 2022). Multiple studies have shown that lactoferrin (Lf) is an anabolic factor (Ying et al., 2012). Lf is a bioactive iron binding globular protein, representative of the transferrin family (Pall & Roman 2020, Pall et al., 2023). In addition to the antioxidant potential, Lf also has anti-inflammatory, immunomodulatory, analgesic antibacterial, antiviral, antifungal and anticancer potential (Hao et la., 2019; Pall & Roman 2020).

Lf is present in various secretions and tissues (Atsushi, et al., 2000), including saliva, milk, colostrum, saliva, uterine secretions, semen,

gastrointestinal fluid (Pall & Roman 2020). In the body, Lf circulates approximately at a concentration of 2–7 μg/mL, being a promoter of bone growth, acting as a growth factor for the proliferation of osteoblasts and having an inhibitory effect on osteoclasts (Amini & Nair 2011, Yin et al., 2020).
In this context, the aim of present study was to evaluate the osteoinductive potential of Lf on MSCs isolated from equine synovial fluid.

MATERIALS AND METHODS

Synovial fluid derived MSCs isolation

This research was approved by the Bioethics Committee of the Faculty of Veterinary Medicine Cluj-Napoca. Synovial fluid was harvested from the metacarpophalangeal joints during arthroscopic procedure from a healthy horse.

MSCs cells isolation

Synovial fluid samples was mixed with propagation medium DMEM/F12 (Gibco Life Technologies, Paisley, UK),) supplemented with 10 % of fetal bovine serum (FBS, Sigma-Aldrich, St.Louis, MO, USA) and 1 % antibiotic-antimycotic (Sigma-Aldrich, St.Louis, MO, USA). Culture plates were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. After 72 h, non-adherent cells were removed, and the culture medium was replaced. The cultures were monitored daily, the medium was changed every 2 days, and at a confluence of 70-80% the cells were passaged. After 6 successive passages the cells were evaluated immunophenotypically using flow cytometry.

Immunophenotype characterization

For the evaluation of specific surface markers, the cells were removed from the cultivation surface, and the cell suspension was labeled with anti-human CD44, anti-human CD105, anti-human CD90, anti-human CD45, anti-human CD73 and anti-human CD 34. The specific antibodies were purchased from Becton Dickinson, USA. A concentration of 1×10^5 labeled cells were acquired with flow cytometry (FACS Canto™II, Becton Dickinson, USA). The results were analyzed using FACSDiva Software (Becton Dickinson, USA).

CCK-8 assay

The synovial fluid derived MSCs were cultured in normal propagation medium supplemented with 3 different concentrations of Lf (20, 50, 100 μg/mL). To evaluate the proliferation potential of synovial fluid derived MSCs a concentration of $1x10^5$ cells were seeded in 96-well tissue culture plates in normal propagation medium. After 24 and 48h of incubation, the cells were treated with 3 different concentrations of Lf and incubated at 37°C in a humidified atmosphere supplemented with 5% CO2. Negative control was represented by untreated cells (cells maintained in normal propagation medium). Cell viability was measured using CCK-8 reagent and incubating for 4h at 37°C. For the interpretation of the results the optical density was determined at 450 nm by using a BioTek Synergy 2 microplate reader (Winooski, VT, USA). The obtained results were expressed as relative viability percentage to the negative control (untreated cells). All experiments were performed in triplicate.

Osteogenic differentiation of isolated cells

To evaluate the osteoinductive potential of lactoferrin, MSCs derived from the synovial fluid were cultured at a concentration of 3×10^3 cells/cm^2 in normal propagation medium. After 24 h the culture medium was removed and the specific osteogenic medium (100 nM dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid (all from Sigma-Aldrich, St.Louis, MO, USA) supplemented with three different concentration of Lf (20, 50, 100 μg/mL) was added. The culture medium was changed every three days, and after 7 and 14 days of culture, the concentration of alkaline phosphatase was evaluated.

Alkaline phosphatase evaluation - fluorimetric assay

Quantitative analysis of ALP was assessed using fluorimetric Alkaline Phosphatase detection kit (Sigma-Aldrich, St.Louis, MO, USA). For evaluation, the samples were collected at 7 and 14 days of culture, respectively. The samples (20 μl) together with the negative control (cells maintained in osteoinductive medium without lactoferrin and

cells maintained in simple propagation medium) were added in microplates with 96 wells. The plates were incubated for 20 minutes at 65°C, after which they were cooled followed by the addition of the diluted fluorescent mixture (1:8). The results were evaluated using Biotek Synergy 2 reader (360 nm excitation and 440 nm emission).

Statistical Analysis

The one-way ANOVA and t-test (GraphPad Prism 8) were used for statistical analysis. The results were expressed as mean ± standard deviation (SD); $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

Synovial fluid derived MSCs cells isolation

Mesenchymal stem cells were derived from synovial fluid from a healthy horse presented for routine analysis. The synovial fluid was incubated in specific propagation medium. After 72 hours the cultures were examined, and the culture medium was changed. The cells attached to the cultivation surface showed spindle morphology along with stellate and round cells. A high percentage of the cells were in suspension. After 5 days, the culture showed a high degree of heterogeneity, and by changing the culture medium, the non-attached cells were removed (Figure 1).

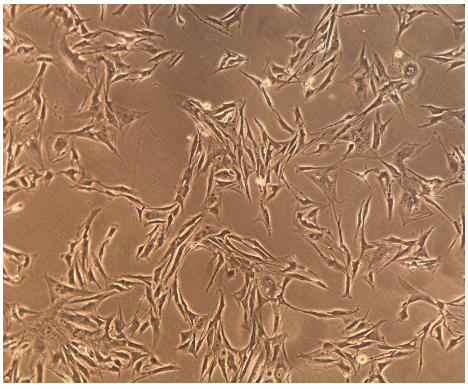

Figure 1. Morphology of cells isolated from equine synovial fluid

At 10 days, the cultures showed a confluency of 80% when the first passage was performed. After the third passage, the culture became homogeneous, the cells showing spindle morphology with pronounced bipolarity, and after the sixth passage, the cells were immunophenotypically characterized.

Immunophenotype characterization

Characterization of cells isolated from synovial fluid was performed using flow cytometry.

The evaluation was carried out according to the standards of the International Society for Cellular Therapy. The isolated cells showed positivity for CD105 (96.5%), CD90 (93.4%), CD44 (99.0%), low expression for CD73 (12.7%) and negativity for CD34, CD45 (Figure 2).

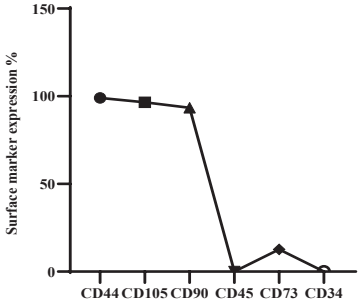

Figure 2. Results of immunophenotype evaluation

CCK-8 assay

To evaluate the proliferative potential of lactoferrin, the propagation medium of MSCs was supplemented with three different concentrations of Lf. Cell viability and the degree of cell proliferation was evaluated by the CCK8 assay. The results suggest that lactoferrin has a stimulating effect on cell proliferation. After 24 hours, the average cell viability of treated cultures was 105.53%±0.41. The highest proliferation rate was obtained at the highest concentration (100 μg/mL) of Lf, the average proliferation rate was 108.47%±4.02 (Figure 3).

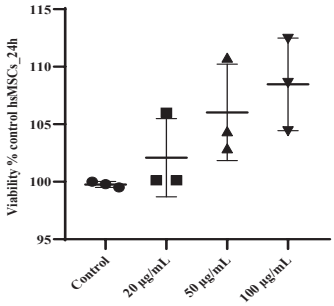

Figure 3. Cell viability rate after 24h of treatment with three different concentrations of human lactoferrin

The increasing tendency of the proliferation rate was maintained even after 48 hours of evaluation. The average of the proliferation rates were 107.30±0.060. Supplementing the propagation medium with a concentration of 100 µg/mL Lf led to an average viability of 111.94%±1.14. The lowest proliferation rate was observed in the cultures treated with 20 µg/mL of Lf. All results were compared with the control culture (Figure 4).

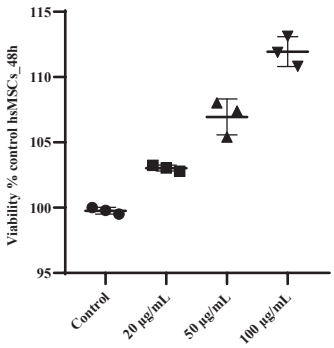

Figura 4. Cell viability rate after 48h of treatment with three different concentrations of human lactoferrin

Through the global analysis of the results, it can be concluded that lactoferrin has a stimulating effect on cell proliferation, and it is dose dependent.

Osteogenic differentiation of isolated cells

To demonstrate the osteoinductive potential of lactoferrin, cells derived from equine synovial fluid were treated with the simple osteogenic medium and the osteogenic medium supplemented with lactoferrin in three different concentrations. At 7 and 14 days, respectively, the cell lysates were tested for the evaluation of ALP activity. The results showed higher levels of ALP in cultures treated with lactoferrin compared to the osteogenic medium and simple propagation medium. 7 days after differentiation, the highest level of ALP was recorded in cultures treated with 100 µg/mL of lactoferrin, followed by those treated with 50 µg/mL of lactoferrin (Figure 5).

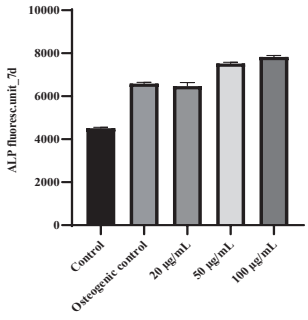

Figure 5. Alkaline phosphatase level evaluated after 7 days of treatment of MSCs with osteogenic medium and lactoferrin

Analyzing the results after 14 days, the ALP levels showed a slight increase, but these increases were not statistically significant compared to the previous evaluations. In this evaluation, similar to the previous ones, the highest values of ALP in the evaluated cell lysates were recorded in the case of cultures treated with the highest concentration of lactoferrin (Figure 6).

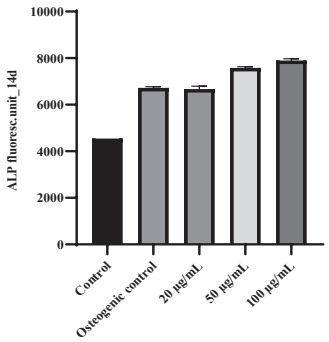

Figure 6. Alkaline phosphatase level evaluated after 14 days of treatment of MSCs with osteogenic medium and lactoferrin

Our results demonstrate the potential of lactoferrin to increase the cells viability of MSCs. Similar studies demonstrate this potential; Ryu et al (2017) indicates that lactoferrin potentiates cell proliferation, which is dose-dependent and occurs through the MAPK signal pathway (Ryu et al., 2017). An important characteristic of MSCs is their multilineage differentiation potential. In the body, this differentiation potential is controlled by several mechanisms and is dependent on the intervention of specific growth factors. *In vitro*, this potential can be demonstrated through various substances, either synthetic or natural. In our experiment we chose lactoferrin because our preliminary studies demonstrate the multiple capacity of this bioactive globular protein (Pall et al., 2023). Multiple studies demonstrate that osteoblast differentiation is a crucial stage in osteogenesis (Spi et al., 2020). To demonstrate osteogenesis, the evaluation of ALP activity is an important indicator (Liu et al., 2015) and is expressed at the beginning of the mineralization process. In the present study, ALP activity was evaluated in cell lysate in two stages of directional differentiation (early and intermediar stage). The studies of Zhang et al. (2014) demonstrated that the oral administration of Lf in ovariectomized rats acted preventively on the reduction of bone loss due to estrogen deficiency and contributed to the improvement of bone microarchitecture. Similar *in vitro* studies demonstrated that Lf promotes osteoblast proliferation and survival, but simultaneously has an inhibitory effect on osteoclastogenesis (Cornish et al., 2004).

CONCLUSIONS

Lf is considered an anabolic factor with a demonstrated role in osteogenesis. Our study demonstrated its differentiation potential on mesenchymal stem cells derived from the synovial fluid. But further studies are needed to demonstrate the potential mechanisms involved. Due to its antimicrobial, antibiofilm, antitumor, anti-inflammatory potential, Lf from different sources can play an important role in veterinary medicine, for the treatment of diseases with multiple etiologies.

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