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UMBILICAL CORD MESENCHYMAL STEM CELLS CHARACTERIZATION: *IN VITRO* PRELIMINARY ASSESSMENTS FOR CHRONIC WOUND HEALING

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INTRODUCTION

Wound healing follows a complex sequence of events whose failure leads to injuries and chronic wounds, that are a major socioeconomical problem since they are difficult to heal. Conventional treatment includes antibiotherapy, debridement, laser therapy, skin grafting and transplantation, that can lead to pigmentary abnormalities, scars, and recurrent ulcers. Therefore, finding safe and efficient alternative treatments is essential and human umbilical cord mesenchymal stem cells (hUC-MSCs) have demonstrated promising results in chronic wound healing [1-3]. In the present study, hUC-MSCs were used and characterized [4, 5]. The following assays were performed to determine their *in vitro* response: growth curve, cell viability, population doubling time (PDT) and, colony unit assays (CFUs). Additionally, multilineage differentiation and RT-qPCR were also performed.

RESULTS AND DISCUSSION

The CFU assay confirmed the cells' ability to form colonies from single cells (Figure 1). After 14 days of incubation, it was possible to identify an average of 7.5 ± 2.1 colonies obtained from 250 seeded cells, 8.5 ± 2.1 colonies obtained from 280 seeded cells, 15 ± 2.6 colonies obtained from 320 seeded cells and 14.7 ± 4.0 colonies obtained from 380 seeded cells. These values, respectively for 250, 280, 320 and 380 cells seeded, translate into a clonogenicity of 3.0 ± 0.85%, 3.1 ±0.78%, 4.7 ± 0.79% and 3.9 ± 1.06%.

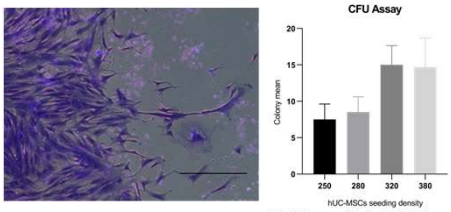


Figure 1: Colony Formation (Cristal Violet Staining) and % of clonogenicity in hUC-MSCs.

The growth curve was established from the cell counts over 6 passages in 30 days. The number of cells quantified increased exponentially in passage number 5 and then decreased rapidly. Regarding the viability, it started to decrease in passage number 3 until passage number 5 and then it increased slightly.

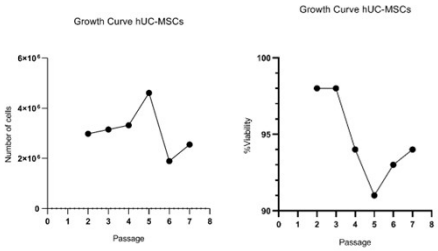


Figure 2: Growth curve and Viability of hUC-MSCs.

In Figure 3 it is possible to observe that the cells have a standard growth pattern, exhibiting the traditional 3 phases: the Lag Phase easily identifiable in the first 2 days; the Exponential Phase, where the cells proliferate fast and at a higher rate, and the Stationary Phase after the 5th day in culture. The typical "S" shape is present and the mean value of PDT for the hUC-MSCs in P6-P7 was 60.1 ± 3.01 hours.

Figure 3: Cell growth of hUC-MSCs over 8 days of culture in P6-P7.

The cells demonstrated the presence of red stained intracellular lipid vacuoles and a change in their morphology to a round shape (**Oil Red** – Figure 4, a), confirming adipogenic differentiation. The cells morphology changed into chondrogenic aggregates, and the extracellular matrix was stained in blue (**Alcian Blue** – Figure 4, b), confirming chondrogenic differentiation. Mineral matrix deposition was observed and evidenced in both staining's by the reddish pigment present in osteocytes and extracellular calcium deposits (**Alizarin Red** – Figure 4, c) and the dark pigment in the cellular surface (**Von Kossa** – Figure 4, d), both confirming osteogenic differentiation. The cells presented no difference in terms of GAGs production (Figure 4, e), however there was difference in terms of ARS production ($p<0.0193$) within the differentiated and undifferentiated groups (Figure 4, f).

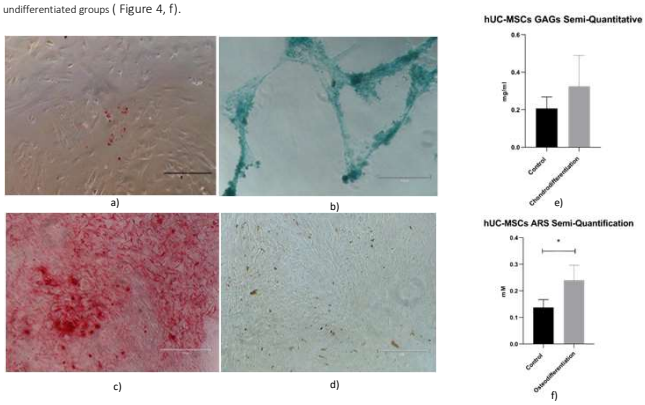
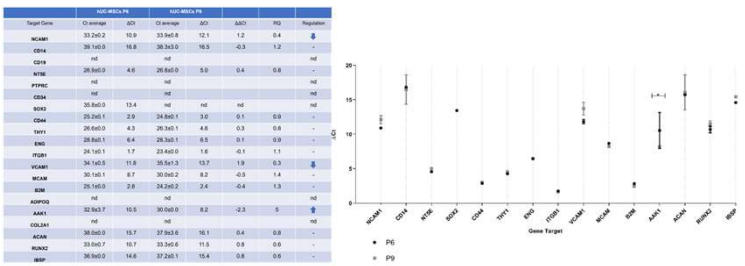


Figure 4: Adipogenic differentiation (a), Chondrogenic differentiation (b), Osteogenic differentiation (c and d). GAGs production (e) and ARS production (f) from hUC-MSCs.

In the RT-qPCR, there was strong expression of the markers CD44, NT5E, THY-1 and ENG (minimum criteria to identify MSCs). The hematopoietic markers were all negative (CD19, CD14, CD34 and PTPRC), except for CD14. There was expression of all the cell proliferation, adhesion and histocompatibility markers (NCAM1, VCAM1, MCAM, B2M and ITGB1). However, there was a down-regulation from P6 to P9 of the NCAM1 and VCAM genes. The amplification of AAK1, ACAN, RUNX2, IBSP demonstrated that these cells can undergo differentiation (adipogenic, osteogenic and chondrogenic). However, ADIPOQ and COL2A1 were not expressed. There was also up-regulation of the AAK1 gene in P9. These results corroborate that these cells are indeed MSCs, but also demonstrate that higher passages are not as stable and can start to lose some of their MSCs characteristics.



Target Gene	hUC-MSCs P6 Ct average	ΔCt	hUC-MSCs P9 Ct average	ΔCt	ΔΔCt	RQ	Regulation
NCAM1	33.2±0.2	10.9	33.9±0.8	12.1	1.2	0.4	↓
CD14	39.1±0.0	16.8	38.3±3.0	16.5	-0.3	1.2	-
CD19	nd		nd				nd
NT5E	26.8±0.0	4.6	26.6±0.0	5.0	0.4	0.8	-
PTPRC	nd		nd				nd
CD34	nd		nd				nd
SOX2	35.8±0.0	13.4	nd	nd	nd		nd
CD44	25.2±0.1	2.9	24.8±0.1	3.0	0.1	0.9	-
THY1	26.6±0.0	4.3	26.3±0.1	4.6	0.3	0.8	-
ENG	28.8±0.1	6.4	28.3±0.1	8.5	0.1	0.9	-
ITGB1	24.1±0.1	1.7	23.4±0.0	1.6	-0.1	1.1	-
VCAM1	34.1±0.5	11.8	35.5±1.3	13.7	1.9	0.3	↓
MCAM	30.1±0.1	8.7	30.0±0.2	8.2	-0.5	1.4	-
B2M	25.1±0.0	2.8	24.2±0.2	2.4	-0.4	1.3	-
ADIPOQ	nd		nd				nd
AAK1	32.9±3.7	10.5	30.0±0.0	8.2	-2.3	5	↑
COL2A1	nd		nd				nd
ACAN	38.0±0.0	15.7	37.9±3.6	16.1	0.4	0.8	-
RUNX2	33.0±0.7	10.7	33.3±0.6	11.5	0.8	0.6	-
IBSP	36.9±0.0	14.6	37.2±0.1	15.4	0.8	0.6	-

Figure 5: Ct, ΔCt, ΔΔCt and RQ values for all genes under study for P6 and P9 hUC-MSCs. nd= non-defined. ΔCt Values for the different genes under study in hUC.MSCs for P4 and P6. Higher delta-CT values represent lower expression (mean±SEM).

CONCLUSION

The main purpose of the present study is the application of hUC-MSCs and their derived secretome *in vivo* in preclinical and clinical trials for chronic wound healing. Therefore, it is important to characterize these cells and, the assays performed so far have established that the hUC-MSCs are indeed MSCs and are adequate to be used in further tasks. The next step will be to study their therapeutic potential for the treatment of chronic wounds, through the characterization of their secretome profile and their application in animal models of skin injury.

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