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Preservation of Biological Characteristics of Warthon's Jelly Stem Cells Post-Cryopreservation

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ABSTRACT. Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) are multipotent, with low immunogenicity and great potential to regenerate tissues, which makes them candidates for their application in cell therapy. Despite scientific advances, their conservation still limits extensive use. This study aims to determine the preservation of genotypic and phenotypic properties of WJ-MSCs that experienced cryopreservation after thawing. WJ-MSCs divided into 3 groups were used: freshly thawed cells (FTC), thawed cells acclimated for 24 h post-thaw (TCA) and non-cryopreserved cells from fresh culture (FC). Expression of *NANOG, SOX2, OCT4* and *HMOX-1* genes was assessed by real-time PCR and phenotyping by flow cytometry and Short Tandem Repeat (STR). For phenotypic evaluation, differentiation media towards osteocytes, chondrocytes and adipocytes were used. Expression of *NANOG, SOX2* and *OCT4* pluripotency genes was conserved. Although, for *SOX2* and *OCT4*, it was lower in FTC when compared to FC. Expression of *NANOG* and *SOX2* genes was recovered in TCA. The anti-inflammatory and immunoregulatory gene *HMOX-1* was not significantly affected in the TCA when compared to FC. All WJ-MSCs were able to differentiate into osteogenic, adipogenic and chondrogenic lineages, they retained their immunophenotype, without presenting mutations in microsatellites evaluated by short tandem repeats (STRs).These data suggest that WJ-MSCs tend to maintain their pluripotency and self-renewal capacities, which are more noticeable after a 24-hour acclimation period.

Key words: Cryopreservation; *NANOG, SOX2; OCT4* and *HMOX-1*; Wharton's jelly

INTRODUCTION

Stem cell therapies have arisen in the scientific medical field as a promising option for a wide spectrum of chronic, incurable or difficult-to-treat diseases such as cancer (Alidadi et al., 2024), infectious (Hussein et al., 2022), cardiovascular (Bakinowska et al., 2024), musculoskeletal diseases (Vaish and Vaishya, 2024), neurodegenerative (Brun et al., 2024) and movement disorders, among others. FDA (food and drug administration) has approved the use of umbilical cord blood stem cells (UCB-SC) for being an excellent source of stem cells for transplants, and hematological malignancies such as leukemia, lymphomas or myelodysplasia (Pochon et al., 2022). WJ-MSCs have immunomodulatory, anti-inflammatory, anti-apoptotic properties, they have the ability to differentiate into cells of mesenchymal origin. Furthermore, since they are obtained from the umbilical cord, they overcome bioethical barriers and their harvesting does not pose a risk for patients, which leads to a good cost/risk/benefit ratio (Le Saux et al., 2020; Antebi, 2018).

Stem cells are specialized cells that have the ability to self-renew, retain their biological properties and differentiate (ability to turn into different types of mature cells). They are classified according to their origin: embryonic stem cells, which have the ability to form all three germ layers (ectoderm, endoderm and mesoderm), and adult stem cells, that are found in different types of tissues or organs, having the ability to differentiate into at least one lineage. According to their differentiation potential, they are classified into totipotent, pluripotent, multipotent and unipotent cells (Lovell-Badge et al., 2021).

WJ-MSCs have established criteria for their identification including adherence to plastic under standard culture conditions, morphology similar to fibroblasts, potential to differentiate into three lineages (adipocyte, chondrocyte, and osteoblast). They must express antigenic surface markers such as CD73, CD90 and CD105, Additionally, to express high levels of CD 29 (β1 integrin) and CD44 (hyaluronan receptor) and to be negative for hematopoietic and endothelial antigens CD45, CD14, CD20, CD34 and HLA-DR.

Their differentiation and immunoregulation capacity can be demonstrated with the expression of several genes, such as *NANOG, SOX2* and *OCT4*, which interact with each other to form transcriptional complexes that regulate their capacity for self-renewal and pluripotency; the absence of these genes triggers cell differentiation. Through the expression of these genes, it has been possible to reprogram adult cells and convert them into induced pluripotent stem cells (iPSCs), managing to maintain them in a totally undifferentiated state (Bura et al., 2014; Koike, 2014; Zhang, 2016).

NANOG gene regulates pluripotency and self-renewal of stem cells by upregulation of key transcription factors such as *OCT4* and *SOX2*, which in turn, activate a network of genes. Inhibiting *NANOG* gene facilitates the action of genes responsible for cell differentiation (Ângelo et al., 2012). *OCT4* and *SOX2* are essential during embryogenesis. Mutating or interrupting *OCT4* expression disables the inner cell mass of blastocysts to differentiate into the epiblast and yolk sac. Preventing *SOX2* expression inhibits the formation of the trophectoderm leading to embryo's death due to its inability to implant in the endometrium (Assadollahi et al., 2019).

HMOX-1 gene has anti-inflammatory and immunomodulatory activities, it codes for enzyme

Heme Oxygenase-1, thus, participating in Heme metabolism, which gives it antioxidant (Raffaele, 2016), anti-apoptotic and cytoprotective properties. In addition it has been shown that inactivation of this gene inhibits angiogenesis (Zeng et al., (2010). Its immunoregulatory function decreases Th2 and Th17 lymphocytes activity through STAT3-SOCS3 pathway (Lin et al., 2020; Wang et al., 2019). Other *in vivo* studies have shown that *HMOX-1* is key to decrease liver transplant rejection through Treg and NK cell regulation (Hu, 2019).

These cells are cultured to be administered or cryopreserved in liquid nitrogen and 10% dimethyl sulfoxide (DMSO). The purpose of cryopreservation is to maintain their viability by slowing down metabolic processes during their long-term storage (Arutyunyan, 2021) preserving the differentiation potential of MSC (Xu et al., 2021), to be subsequently thawed and administered to patients. However, information on preservation of their functions after thawing is limited (Antebi et al., 2019); some studies state that cryopreservation can negatively affect the function of these cells after this process (Xu et al., 2012; Zhang et al., 2021; Davies et al., 2014).

In the process that leads to WJ-MSCs cryopreservation, there may be changes that alter the capacities and potentialities of these cells, because it interacts with them directly stopping cell cycles (Shivakumar et al., 2015; Sun, 2020; Jang et al., 2018) and indirectly by cryoprotectants, some of which have evidence supporting their function as epigenetic modulators (Alizadeh et al., 2016; Bozkurt et al., 2019), which would imply the loss of its pluripotency, regeneration, anti-inflammation and immunoregulation. This study aimed to evaluate preservation of genotypic characteristics of WJ-MSCs in fresh cell samples (from cultures) and in post-cryopreservation cells by studying *NANOG, SOX2, HMOX-1* and *OCT-4* genes, and to assess their ability to differentiate to osteogenic, adipogenic and chondrogenic lineages.

MATERIALS AND METHODS

In order to perform different experiments, umbilical cord mesenchymal stem cells (Warthon's jelly), supplied by Stem Cell and Biotechnology Center Regencord were used. Cells were classified into 3 groups as follows: i) non-cryopreserved cells called fresh cells (FC), ii) cryopreserved cells, thawed and analyzed immediately; called freshly thawed cells (FTC) and iii) cryopreserved cells, thawed and acclimated for 24 hours at 4°C, called acclimated thawed cells (TCA)

WJ-MSCs were cultured until passage 6 (P6) and cryopreserved in 2 mL cryovials using 10% Dimethyl sulfoxide (DMSO), 65% plasmalyte and 25% albumin as cryoprotective media and, subsequently, they were stored in vapor-phase nitrogen at -196°C for 3 months. Vials were thawed rapidly by immersion in a 37°C water bath for 120 seconds and then washed in saline solution and centrifuged to remove the cryopreservation solution. Subsequently, cells were divided into freshly thawed cells (FTC) and acclimated thawed cells (TCA) and compared with fresh cells (FC) according to the protocol used by Antebi B.(22). The following parameters were evaluated:

I. Differentiation potential

II. Preservation of genetic identity

III. NANOG, SOX2, HMOX-1 and OCT-4 gene expression

IV. Preservation of immunophenotype

Differentiation potential

Differentiation potential was evaluated by inducing differentiation of FC, FTC and TCA cells towards osteogenic, chondrogenic and adipogenic lineage, following the corresponding StemPro protocols.

Preservation of genetic identity

Evaluation of cell's genetic profile of the three groups was carried out. Filter paper was impregnated individually with about 500 000 cells of the FC, FTC and TCA samples; after drying, 2 punches were cut and deposited in PCR tubes. VeriFiler™ Plus PCR Amplification Kit which contains 23 autosomal STRs markers was used following the manufacturer's instructions. GA-3500 genetic analyzer (Applied Biosystems) was used. Concordance in genetic profiles between groups and the absence of point mutations were evaluated. Results were analyzed using GeneMapper™ ID-X 1.6 software.

Gene expression

For evaluating *NANOG, SOX2, HMOX-1* and *OCT-4* gene expression of cells supplied by the Regencord laboratory, total RNA was extracted from FC, FTC and TCA of WJ-MSCs using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. mRNA expression of *NANOG, SOX2, HMOX-1* and *OCT-4* genes was determined using the *18S* gene as an endogenous control by Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) and, using SmartCycler® Real Time System (Cepheid®) and TaqMan® probes. RT-qPCR was carried out in a 25 uL final reaction volume, using TaqMan® RNA-to-Ct™ 1-Step Kit following the manufacturer's instructions and TaqMan® probes for *SOX-2 (Hs01053049_s1), NANOG (Hs 02387400_G1), HMOX-1 (hs01110250 m1), OCT-4* (*Hs00999632_g1*) genes and the *18S* endogenous control *(Hs03929097 g1)*. A thermal profile of 48°C for 15 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, and 60°C for 1 min was used for amplification. Results were automatically analyzed by SmartCycler software. Relative quantification was analyzed using the 2 -ΔΔCT comparative method. All experiments were performed in duplicate.

Preservation of immunophenotype

Analysis of expression markers CD73, CD90, CD105, CD45, CD34, CD11b, CD19, HLA-DR was performed as part of the minimal criteria for defining MSC according to the International Society for Cellular Therapy (ISCT). Flow cytometry was performed at the Laboratory of Molecular Biology and Biotechnology of Technological University of Pereira according to standard protocols.

STATISTICAL MANAGEMENT

Data were expressed with mean ± standard deviation to measure expression of genes encoding *NANOG, SOX-2, OCT-4, HMOX-1* by the relative quantification method (ΔCT) and are presented as descriptive statistics in the form of graphs of the different populations FC, FTC and TCA.

RESULTS

Differentiation potential

Cryopreserved WJ-MSCs, subsequently cultured and, exposed to different media differentiated into chondrogenic, adipogenic and osteogenic lineages, which is evidenced by the morphological changes that these cells underwent. Fixation methods used in adipogenesis allowed the researchers to observe the presence of adipose vesicles. In chondrogenesis, more stained nucleus cells were visualized, in contrast to the pale blue cytoplasm. In osteogenesis, the stained bone matrix is visible (Figure 1).

Preservation of genetic identity

In the 22 markers that were analyzed, 100% cellular concordance was found, between FC, FTC and TCA all coming from WJ-MSCs (Table 1).

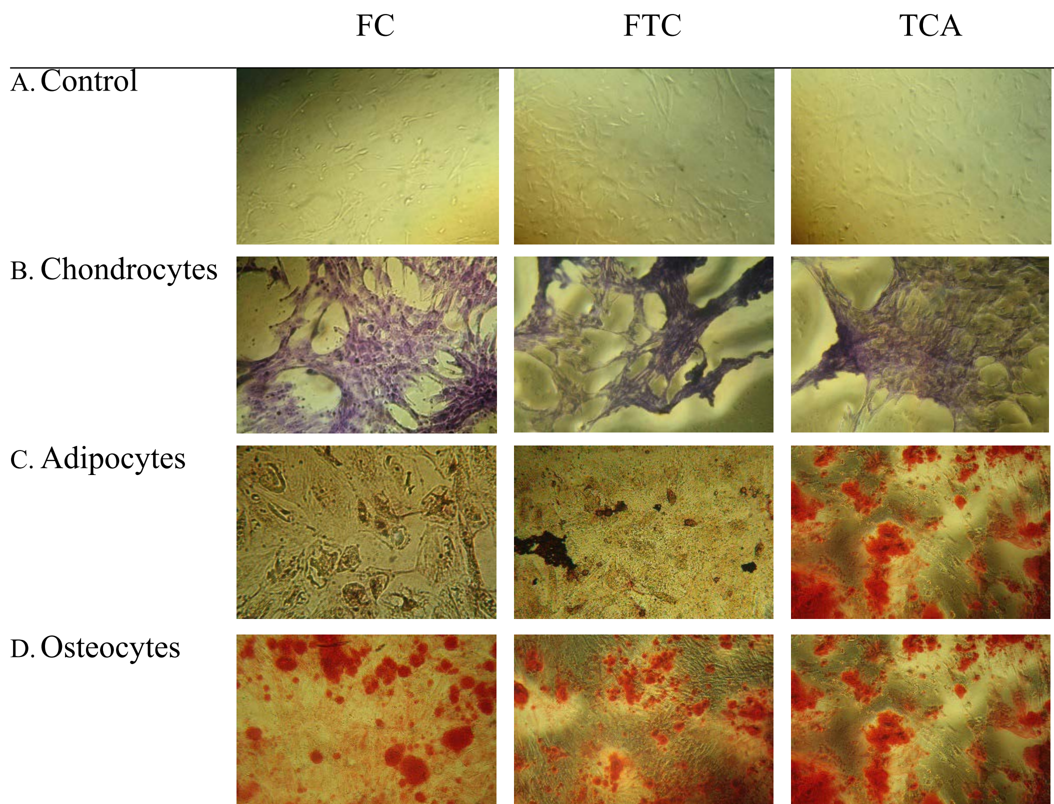

Figure 1. Comparative photos taken at 10x using Olympus reverse microscope of WJ-MSC in culture, before and after being subjected to different differentiation media. A. Control cells without exposition to differentiation medium. B. Cells exposed to chondrogenic differentiating medium. C. Cells exposed to adipogenic differentiating medium. D. Cells exposed to osteogenic differentiating medium.

Table 1. Comparison of STR markers between Fresh (FC), freshly thawed (FTC) and acclimated (TCA) Cells.

STR	**FC**	**FTC**	**TCA**	**Result**
D3S1358	15	15	15	Concordant
vWA	14 / 19	14 / 19	14 / 19	Concordant
D16S539	9 / 11	9 / 11	9 / 11	Concordant
CSF1PO	12	12	12	Concordant
TPOX	8 / 11	8 / 11	8 / 11	Concordant
D8S1179	13 /16	13 /16	13 /16	Concordant
D21S11	28 / 30	28 / 30	28 / 30	Concordant
D18S51	16 / 19	16 / 19	16 / 19	Concordant
D2S441	10 / 14	10 / 14	10 / 14	Concordant
D19S433	14 / 15	14 / 15	14 / 15	Concordant
TH01	6 / 9.3	6 / 9.3	6 / 9.3	Concordant
FGA	22	22	22	Concordant
D22S1045	15 / 17	15 / 17	15 / 17	Concordant
D5S818	11 / 12	11 / 12	11 / 12	Concordant
D13S317	11 / 12	11 / 12	11 / 12	Concordant
D6S1043	18 / 21.3	18 / 21.3	18 / 21.3	Concordant
D10S1248	14 / 15	14 / 15	14 / 15	Concordant
D1S1656	17.3	17.3	17.3	Concordant
D12S391	22 / 23	22 / 23	22 / 23	Concordant
D2S1338	18 / 25	18 / 25	18 / 25	Concordant
PENTAD	11 / 14	11 / 14	11 / 14	Concordant
Amelogenin	X	X	X	Concordant

NANOG, SOX2, HMOX-1 and *OCT-4* gene expression

Responses to cryopreservation of Pluripotency, self-renewal and anti-inflammation properties of WJ-MSCs were evaluated by the expression of *NANOG, SOX2, OCT4* and *HMOX-1* genes. As shown in Figure 2, *HMOX-1* gene expression decreases in cells preserved cryogenically, thus it decreased both in FTC ($p<0.001$), however it was not significative in the TCA ($p<0.052$) when compared to FC.

NANOG gene maintained its expression in FTC ($p=0.9$), and it significantly increased in TCA ($p<0.00001$) comparing to FC. When evaluating *SOX2* gene, downregulation was observed in the FTC ($p<0.001$) and increased its expression in TCA ($p=0.06$) when compared to FC. *OCT4* gene showed significant downregulation in both FTC ($p<0.0002$) and TCA ($p<0.0001$) in comparison with CF (Figure 3).

Preservation of immunophenotype

Markers CD11b, CD19, CD34, CD45, HLA-DR were less than 2% in all groups, while markers CD73, CD90, CD105 were greater than 95% for all groups (Table 2).

Table 2. Immunophenotypic markers for WJ-MSC performed by flow cytometry.

Sample	CD11b, CD19, CD34, CD45, HLA-DR %	CD73%	CD90%	CD105%
TCA	0.3	98.9	99.9	98.9
FTC	0.3	99.9	100	98.8
FC	0.2	99.8	99.9	97.4

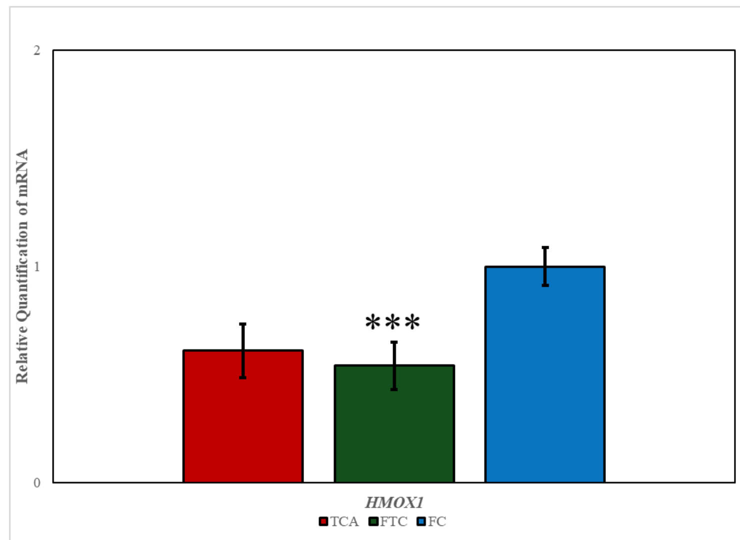

Figure 2. *HMOX-1* gene expression decreased in both FTC and TCA compared to FC. However, this decrease was statistically significant only in the FTC (p<0.001).

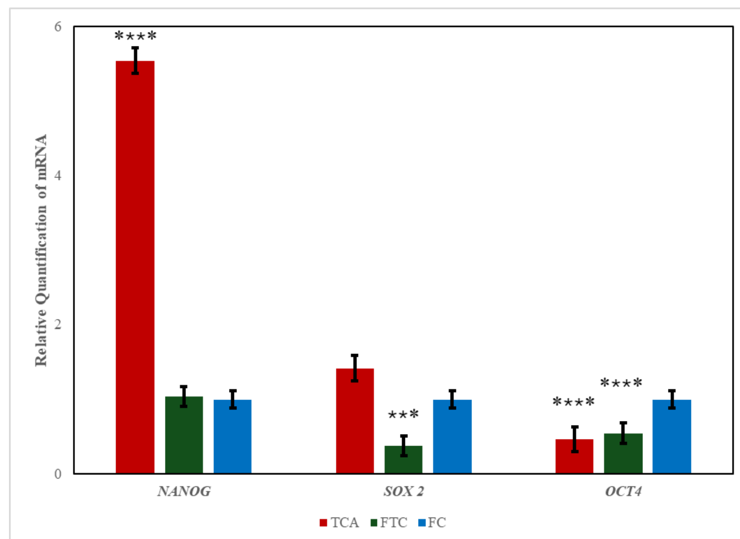

Figure 3. FTC decreased *SOX2* (p<0.001) and *OCT-4* (p<0.0002) expression, but it is observed that in TCA, *SOX2* recovered it. When observing NANOG, there is evidence of an increase in its gene expression in TCA (p<0.00001).

DISCUSSION

WJ-MSCs have the potential to treat a large number of diseases and even to repair organs, since they have multiple properties of pluripotency, self-renewal, anti-inflammation, immunomodulation and generation of growth factors, making them ideal candidates for clinical applications in regenerative medicine. Normally, WJ-MSCs are preserved using DMSO at -196°Celsius in liquid nitrogen for months and even years. However, despite their widespread use, there is emerging evidence that this procedure changes the biological properties of the cells to some degree (Davies et al., 2014; Tam et al., 2020). When evaluating the expression of some of the transcription factors NANOG, SOX2 and OCT4, it was observed that in WJ-MSCs that were exposed to the conventional cryopreservation methods, the expression of *OCT4, SOX2* and *NANOG* genes was not impeded, as was evident after 24 hours of acclimation.

NANOG gene regulates pluripotency and self-renewal of stem cells by upregulation of key transcription factors such as OCT4 and SOX2, which in turn, activate a network of genes. It was found that expression levels did not vary in FTC and increased in TCA as described by Aziz *et al*, where upregulation of this gene occurred when evaluating six different cryopreservation methods for adipose-derived MSCs (Yong et al., 2015). Antebi *et al.*, 2018 (Antebi, 2018) found that this gene decreases its expression in FTC and TCA. However, these samples were obtained from bone marrow. Other authors reported that *NANOG* expression was maintained during immediate thawing (Shivakumar et al., 2015; Sun, 2020).

In our study, a decrease in the expression of *SOX2* and *OCT4* was observed in FTC. However, it is evident that a 24-hour acclimation period leads to the recovery of *SOX2* expression at levels higher than those of FC, although it was not statistically significant, which is consistent with findings of other authors (Yong et al., 2015; Jang, 2018). It has been previously reported that expression of both genes in WJ-MSC is not altered after cryopreservation, it should be noted that in these articles the rt-qPCR evaluation was performed after a new culture period which could influence the results obtained (Shivakumar et al., 2015; Shivakumar et al., 2016).

The interaction between *NANOG, SOX2* and *OCT4* has been the object of continuous study and its behavior has not yet been completely elucidated, bimodal models have been proposed in which increases of *NANOG* expression would regulate the behavior of *SOX2* and *OCT4*. This behavior was observed in FTC and partially in TCA of this study, where the sustained decrease of *SOX2* could lead to the upregulation of *NANOG*. This behavior was not found in *OCT4,* which maintained its decreased expression despite acclimation. Current knowledge indicates that this circuit is constantly fluctuating, which could explain the divergence between the findings from different authors (Yu, 2018; Sasai et al., 2013).

Expression of the anti-inflammatory and immunoregulatory gene *HMOX-1* was evaluated after cryopreservation, finding an expression that is statistically significant decreased in FTC, but not in the TCA in which there was no statistically significant difference compared to the FC. Regarding the expression of this gene, (Antebi et al., 2018) found upregulation of the gene expression post cryopreservation. Literature is scarce relative to *HMOX-1* expression; therefore, further studies are necessary in order to specify its activity in stem cells post-cryopreservation.

As to Immunophenotyping, WJ-MSCs are known to express CD73, CD90 and CD105. Analysis of surface markers before and after cryopreservation by flow cytometry indicated that WJ-MSCs from all groups were positive for the surface markers CD 73, CD90, CD105; and negative for

CD 14, CD20, CD34, CD45, HLA-DR (Bozkurt, 2019; Tam et al., 2020; Yong et al., 2015). Hence, immunophenotype was not altered by cryopreservation in accord with other reports (Antebi et al., 2019; Shivakumar et al., 2016).

Concordance with markers used internationally in the microsatellite study (STR) between cryopreserved cells against FC, showed that this conservation method does not cause mutations in cells after thawing them, which indicates that they retain their genome after cryopreservation, which is identical to that of the original cells. In addition, all WJ-MSCs groups retained their differentiation potential. This is evidenced by the multilineage differentiation to osteocytes, chondrocytes and adipocytes as demonstrated by other studies, which found no alteration of this capacity in umbilical cord MSC using different cryopreservation protocols (Shivakumar et al., 2015; Isildar et al., 2019).

Currently, there are clinical trials with previously cryopreserved WJ-MSCs, demonstrating that their use is safe and has therapeutic benefits. A study carried out in 2021, involving 112 people with hematological disorders and who received hematopoietic stem cells from bone marrow, showed no differences in the use of bone marrow grafts without cryopreservation versus cryopreserved cells in neutrophil engraftment(37). Soder *et al.* in 2020 (Soder et al., 2020) stated that the intravenous application of thawed WJ-MSCs to treat graft-versus-host disease has proved to be safe and they suggested that its effect is similar to that of other immunosuppressive drugs used in this pathology. In a study conducted by Albu *et al*, (Albu et al., 2021) in which 10 patients with spinal injury underwent intrathecal transplantation of WJ-MSCs, it was found that their use was safe and led to the improvement of neurological sequelae. The potential therapeutic use of WJ-MSCs is not limited to their direct application. Other methods are also proposed to benefit from their qualities through the use of their exosome, small vesicles from stem cells that transport cell products among other anti-inflammatory cytokines, mRNA and proteins, that can be isolated and used in clinical practice (Sengupta et al., 2020).

The results obtained in our study suggested that DMSO cryopreservation method used in WJ-MSCs does not compromise the function of pluripotency and self-renewal of genes. Regarding to the anti-inflammatory and antioxidant gene *HMOX-1*, its expression was not significantly affected after 24 hours of thawing. No alterations in their immunophenotype or mutations in the evaluated STR were evident and they maintained their differentiation potential; therefore, WJ-MSCs retain their biological properties post-cryopreservation.

This study has limitations, like the fact that WJ-MSCs were obtained from a single donor. Donor and batch variability are recognized as factors that influence gene expression studies. Therefore, further verification of these results in samples collected from multiple donors and with different cryopreservation durations are necessary. In addition, there are genes reported with similar functions that were not evaluated in our study. Thus, other studies on kinetic expression for longer periods are required.

CONCLUSIONS

The findings of this study suggest that after thawing and a period of acclimation, WJ-MSC maintain the biological properties of the cells from which they are derived, in addition to other qualities such as their constant availability and ease of acquisition, making WJ-MSC an ideal candidate to be used in future clinical research and stem cell therapies. To fully evaluate the immunoregulatory and anti-inflammatory capacity of cryopreserved stem cells, further studies are needed to analyze of their behavior against stimuli *in vitro* and *in vivo*.

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ETHICAL/LEGAL ASPECTS

This project was approved by the Bioethics Committee of Technological University of Pereira and classified as "risk-free research", since it does not involve humans and was performed on cells from umbilical cord fragments, tissues that are usually thrown away.

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: study conception and design: Carlos Gonzalo Arteaga, Gloria Inés Hincapie, Julieta Henao; data collection: Carlos Gonzalo Arteaga, Gloria Inés Hincapie, Leonardo Beltran, Jainer Aranzazu; analysis and interpretation of results: Carlos Gonzalo Arteaga, Gloria Inés Hincapie, Julieta Henao, Jainer Aranzazu; draft manuscript preparation: Carlos Gonzalo Arteaga, Gloria Inés Hincapie, Julieta Henao, Jainer Aranzazu. All authors reviewed the results and approved the final version of the manuscript.

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