

# A novel method for maintaining the stability of freshly cultured Mesenchymal Stem Cells in clinical grade injection ready state without cryopreservation

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## Research

**Keywords:** mesenchymal stem cells (MSCs), storage, plasma

**Posted Date:** May 13th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-379388/v1>

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# Abstract

## Background

Mesenchymal Stem Cells (MSCs) are multipotent cells with low immunogenicity, and dynamic tissue repair potential, which explains the overwhelming attention they have attracted in regenerative therapy. One notable challenge in MSCs therapy is bench to bed timeline of freshly cultured MSCs; it does not exceed 24 hours. For use after 24 hours, MSC need to be cryopreserved, a process that preserves the cells for years, but is costly and damaging. Here we introduce a method to extend the bench to bed lifetime of MSCs up to 4 days without the high cost and cell damaging effects of cryopreservation. Our method uses human plasma as medium.

## Methods

MSCs of 12 tissue samples – 4 adipose, 4 bone marrow and 4 Wharton's jelly- were cultured and expanded under standard conditions. After the cell harvest, each sample was suspended in human plasma. Cell suspensions were refrigerated ( $5 \pm 3^\circ \text{C}$ ) or stored at room temperature ( $22 \pm 3^\circ \text{C}$ ). During the next 7 days, two tubes (one from each group) were examined every 24 hours to assess MSCs viability and growth potential. On day 3, we assessed MSC 1) differentiation potential to adipocyte and osteocyte tissues 2) surface markers. Results were analyzed by computing the overall mean and applying the independent-samples t-test to those means.

## Results

The sample means for both cell expansion and cell viability were compared between the two “fridge” and “room temperature” groups. Although there was a gradual decrease in cell growth potential between the cells stored for 1 day to those stored for 7 days, we show more than 80% of the cells remain alive for up to 4 days of storage in both groups. The cells reached 80% confluency in under 20 days for all samples stored up to 4 days. No significant differences were observed between the two groups (room temperature and fridge stored). The differentiation potential to adipocyte and osteocyte tested on day 3 were positive in all samples. The analysis of cell surface markers tested on day 3 were positive for CD90, CD105, CD73 and negative for CD34, CD45 and HLA-DR.

## Conclusion

We present a method of MSC culture medium using human plasma that can preserve their viability and growth potential for up to 4 days in both room and refrigerator temperatures without losing their stemness characteristics (we recommend use of  $5 \pm 3^\circ \text{C}$ ). This novel method will allow rapid expansion

and therapeutic use of MSCs. Since the cells can be maintained in clinical grade, injection ready state for several days, they can be transported across the globe.

## Introduction

Mesenchymal stem cells (MSCs) have an innate capacity for multipotent differentiation, giving them invaluable potential for clinical applications, therapeutic uses and regenerative medical practice. MSCs use in clinic was reported in 1995 (1) and has been actively researched ever since (2, 3). MSC have been harvested and expanded in culture for treatment of many degenerative diseases both autologously and allogeneically. To therapeutically use these cells for patients, MSC must be kept in a clinical grade medium after expansion and harvesting. This medium must maintain the biological characteristics of the cells until they are administered to the patient. The standard method suggested and used to date is sterile clinical-grade injectable normal saline with a small percentage of human serum albumin (2-5 % based on various studies) (4-6). This cell suspension must be injected within 24 hours, otherwise MSCs begin to lose their biological characteristics. The standard method for long term storage of these cells is cryopreservation. Cryopreservation is a valuable method in stem cell research and regenerative medicine. However, there are some risks associated with cryopreservation, which include:

1. Decreased survival rate: Cells must be taken from a metabolic state to a frozen state for storage and then from a frozen state back to a metabolic state for use. During this transition, intracellular ice crystals could form and damage the cell (7, 8).
2. Use of DMSO: Using DMSO when cryopreserving the cell will prevent formation of intracellular crystals. However, DMSO is cytotoxic for cells in their metabolic state (when they are no longer frozen).
3. This is a time consuming and expensive method. The freezing and thawing procedure causes considerable decline in cell viability.

Since 1924, when MSC was discovered, they have garnered substantial attention. This does not come as a surprise, because MSC have the potential to differentiate into mesodermal cell lineages such as adipocytes, osteocytes and chondrocytes. They can also trans-differentiate into cells originating from the ectodermal and endodermal lineage including neural cells and hepatocytes (12, 13). These cells have the potential for immunomodulation, anti-inflammatory regulation, growth factor production and anti-apoptotic effects (14-17). To date, most therapies based on stem cells use cryobanked cells. Recent studies suggest the freeze-thawing process of cryopreserved cells could adversely affect their therapeutic potential compared to freshly cultured cells (18). In 2000, Bennet W et al (19) reported the results of their islet transplants in type I diabetic patients. They discovered when islet cells are exposed to blood, instant blood mediated inflammatory reaction (IBMIR) is triggered. This is a phenomenon characterized by activation of the complement system, coagulation and platelet consumption (20). This means injected stem cells become targets for lysis via the complement system and thus their therapeutic effect is diminished. Moll et al (21) has compared the therapeutic, immunomodulatory and blood regulatory potential of freeze-thawed versus freshly cultured MSCs. They demonstrated that in-vitro, freeze-thawed

MSCs are more prone to the IBMIR phenomenon and lysis via the complement system compared to freshly cultured MSCs. Freeze-thawed MSCs displayed a diminished response to pro-inflammatory cytokines and decreased anti-inflammatory cytokine secretion profile. Additionally, they studied the expression and enzymatic activity of immunomodulatory factor IDO. IDO is an immunosuppressive enzyme that activates Treg cells. Twenty four hours after stimulation, expression of this enzyme was dramatically higher in freshly cultured MSCs compared to their freeze-thawed counterpart. However, 7 days after stimulation with IFN- $\gamma$ , the activity and levels of secreted IDO was comparable between the two therapeutic groups. Results of patient follow up were as follows: 7 freeze-thaw injected patients showed 71% response, while the 5 fresh culture MSC injected patients, had 100% response. The authors observed no long-term engraftment issues for either group. We believe that freshly cultured MSCs are preferable to freeze-thawed MSCs and suggest that when cryopreserved cells are used in clinical trials, cells should be cultured until their biological activities have been restored before being administered to patients. Other studies have shown that during the freeze-thaw process, 20-30% of cell viability is irreversibly compromised. Notably, freeze-thawed cells have a diminished immunomodulatory potential due to up-regulation of heat-shock response and disrupted IFN- $\gamma$  function (22, 23). Transfusion of Freeze-thawed MSC in mice yields considerably shorter viability compared to freshly prepared MSC. This limitation of thawed cells is not permanent; if cells are cultured for 24 hours after thawing, they regain their potency (24). Therefore, when using MSCs for clinical purposes, freeze-thawed cells should be kept in in-vitro culture conditions until they regain their full potential before in-vivo therapeutic application.

One of the limitations of using fresh MSCs in clinical treatments is their bench to bed timeline. Presently, MSCs can be maintained in normal saline for 24 hours before injection. For use after 24 hours, our only alternative to date is cryopreservation. In spite of its disadvantages, cryopreservation can maintain viability of MSC for years. In this paper, we introduce a method to maintain fully functional cultured MSC at least 4 days, forgoing the expense and cellular damage caused by freezing.

In this study, we used AB+ human plasma medium to maintain freshly cultured cells for several days and compared their biological characteristics to cells maintained in normal saline containing human serum Albumin (HSA) in the fridge and at room temperature.

## Materials And Methods

Mesenchymal stem cells (MSCs) were isolated from 5 bone-marrow, 5 Wharton's jelly of placenta cord and 4 adipose tissue sources: bone-marrow cells were extracted from Multiple Sclerosis (MS) patients and adipose tissue was extracted from Acute Respiratory Distress Syndrome (ARDS) patients who were candidates for mesenchymal stem cell therapy. These samples were taken from a pool of stem cells prepared for therapy. Because the purpose was evaluating the stability of patient cells for patient treatment, we worked on samples taken for this exact use. Placenta cord samples (as allogeneic source for therapeutic use) were taken from infants from cesarean births. Informed consent forms were filled by patients and mothers at time of sample collection.

## Ethical considerations:

The study design and the informed consents for adipose mesenchymal stem cells were approved by the Ethics committee of Baqiyatallah University of Medical Sciences (ethics code: IR.BMSU.REC.1393.32). For bone marrow MSC, approved by Tehran University of Medical Sciences (TUMS) and Shahid Beheshti University of Medical Sciences (ethics code: IR.SBMU.REC.1395.82). Whorton's jelly was obtained from Sina Cell. Sina cell provides questionnaires and informed consent papers for all donors at the time of collection.

**Normal saline method:** 5 bone marrow, 5 Whorton's jelly cord cells and 4 adipose tissue samples were collected for this method. These 14 samples were isolated and expanded using standard methods (25-28). Four million MSCs were isolated from each sample at the time of stem cell therapy. All the cells were from the first 3 passages. Cells were kept in normal saline solution supplemented with 2.5% HSA (prepared by the Iranian Blood Transfusion Organization) and 1U/ml heparin and prepared as an injection ready suspension. We took a sample from this cells suspension to test cell viability at collection time (zero hour). This sample was transferred to two sterile polystyrene tubes, each containing  $2 \times 10^6$  cells/ml. One of the tubes was kept in the fridge ( $5 \pm 3$  degrees) and the other at room temperature ( $22 \pm 3$  degrees). Each sample was tested for viability at 6, 12, 18 and 24 hours after collection time.

**Plasma culture method:** 4 of each of the following samples were collected: bone marrow, Wharton's jelly and adipose tissue. These 12 samples were isolated and cultured using standard methods. 12 sterile tubes were used for each sample. Collected cells were used to evaluate stability of first three passages. We prepared a 25 million MSC cell ( $2 \times 10^6$  cells/ml) suspension in human AB+ plasma medium (prepared by the Iranian Blood Transfusion Organization). We then transferred 1ml of this suspension to each of the 12 sterile tubes. Six tubes were transferred to the fridge ( $5 \pm 3$  degrees) and 6 were placed in room temperature ( $22 \pm 3$  degrees). On days 1 to 5 and day 7, one tube from the fridge and one tube from the room temperature incubator were tested for both cell viability and in-vitro growth potential. Cell surface markers, adipocyte and osteocyte differentiation potential of each sample in both temperature conditions were tested on day 3. Two tubes from the 3 cell sources was tested for differentiation potential at both temperature conditions on day 5.

Each sample was tested for adipocyte and osteocyte differentiation potential and cell surface markers CD34, CD45, CD73, CD90, CD105 and HLA-DR (flowcytometric analysis) at harvest time before it was considered for this study. Differentiation of these cells to adipocyte and osteocyte was confirmed with Oil red O and Alizarin red staining respectively. MSC were identified as cells that were 95% or more positive for CD73, CD90 and CD105 markers and less than 2% negative for CD34, CD45, and HLA-DR markers. Viability tests were performed by day exclusion method using Trypon blue.

To test for growth potential, cells were cultured in medium containing 1% L-Glutamine+ 10% FBS+DMEM-LG without antibiotics in 6-well cell culture plates under GMP grade conditions. Culture medium were replaced with fresh medium every 3 days until cell confluency reached over 80%. Growth potential was

considered positive if the cells reached more than 80% confluency within 20 days of culture. To evaluate the cell surface markers CD34, CD45, CD73, CD90, CD105 and HLA-DR, we use flowcytometric analysis.

To test for stem-ness of these cells, their differentiation potential was evaluated in specific differentiation medium for adipocyte and osteocyte. To confirm the cell differentiation, adipocyte and osteocyte cultures were stained by Oil red O and Alizarin red staining respectively.

## Results

Viability tests for cells suspended in normal saline (normal saline+2.5% HSA+ 1 heparin U/ml) performed at 6 hour intervals (over 24 hours) in fridge and room temperature conditions did not show any significant difference using independent sample T test. Chart 1 shows that percent of viable cells decreases slowly (demonstrated by the mild downward slope) and maintains more than 80% viability for 24 hours. The average viability in fridge and room temperature conditions are 84% and 86% respectively for these samples.

These cells were cultured in adipocyte and osteocyte differentiation medium, they maintained their growth and differentiation capabilities at 18 and 24 hour time points, both in the fridge and at room temperature (Figure 1 &2). We observed no growth in any of the samples after the second day. These results clearly show normal saline suspension cannot maintain these cells for more than 24 hours.

The cells maintained in human AB+ plasma solution (fridge and room temperature conditions) over the period of a week, did not show a significant difference in vitality over time. As you can see in chart 2, vitality of cells in both temperatures had a slight decline each day, but remained above 80% until the fifth day. After day 5, cell vitality dropped to around 70%. Growth potential of cells suspended in human AB+ plasma solution was tested based on confluency (above 80%) at 20 days for both temperatures. We did not observe any significant difference over this period (chart 3). Confluency of 80% or more was noted for cells cultured up to 5 days, however, the growth potential of day 7 cells did not have such favorable results and in some samples they did not have acceptable growth rates.

Vitality and growth potential of cells taken from the 3 different tissue sources and suspended in human AB+ plasma over 7 days was statistically analyzed for multivariate variance using the One-way ANOVA comparisons (post Hoc) test. Comparing vitality potential results from the 3 different sources in both temperatures did not yield statistically significant results. Growth potential of these cells in both temperature conditions showed no significant difference on days 1 and 2. However, after the second day, we observed that cells isolated from adipose tissue had notably better growth rates compared to cells isolated from Wharton's jelly and bone marrow (chart 4).

Results of differentiation from cells maintained in AB+ plasma for both temperature conditions on days 3 and 5, demonstrated these cells maintain their differentiation potential to adipocyte and osteocytes (figure 3 & 4).

Results of cell surface markers were positive for CD90, CD105 and CD73 (more than 95%) and negative for CD34, CD45 and HLA- (less than 2%) (Figure 5).

## Discussion

Stem cell therapy, as a field of study and a promising clinical treatment, is growing exponentially. Among stem cells, MSCs have distinguished themselves as cells with the greatest applied potential because of their unique combination of characteristics including multipotency, accessibility, culture competency and low immunogenicity, immunomodulatory and tissue repair potential (29-31). One of the most important issues in cell therapy is successful maintenance of cells from bench to bedside. It is imperative to have a standard medium suitable for maintaining these cells for an adequate amount of time until they reach the bedside. This must be done without any significant change in the shape or characteristics of these cells before injection. Therefore it is crucial to determine the shelf-life where all their potentials are maintained (26, 32). To date, the best method for long term maintenance of cells and live tissue samples is cryopreservation. In cell therapy today, if we do not want to freeze the cells, we must treat patients on the day cells are harvested. These cells are kept in HSA supplemented normal saline solution and we have shown the extent of their stability in this study (up to 24 hours). Many times it is not possible to treat the patient on the same day due to unanticipated medical issues, distance or other problems for the patient or physician. Under such conditions, the cells must be frozen, but doing so for a few days is costly and will compromise the quality of the cells. Therefore, finding a way to maintain these cells for a few days will be financially *and* medically invaluable. Furthermore, studies show that fresh cells (not frozen previously) yield better results in therapeutic applications (33). In her 2013 report, Maria Thompson showed that freeze-thawed cells are functionally impaired, as demonstrated by the therapeutic effects, compared to freshly isolated cells (34). A study using human hepatocytes after cryopreservation observed metabolic defects in these cells. This study reported that the cryopreserved hepatocytes had lost most of their capacity for adherence to surfaces. Adhesion to surface plays a defining role in successfully transfusing these cells (35). In hematopoietic stem cell transplantation, researchers have reported use of fresh cells (not cryopreserved cells) in transplant patients result in faster engraftment, less neutropenic fever, and shorter hospitalization time. Chinnaduria et al (36) found that cryopreserved MSC adhesive capacity to fibronectin was reduced by 40% compared to freshly cultured MSC. Cryopreserved MSC binding defect compared to fresh MSC was even greater when adhering to endothelial cells (up to 80%). This observation is consistent with the 60% reduction in cytoskeletal F-actin observed in thawed cells. They also showed that while thawed MSC were not detectable after injection, freshly isolated human MSCs were detectable up to 24 hours after injection into murine liver.

One of the important aspects of MSCs is their immunomodulatory function. Many researchers have explored this capacity in freeze-thawed MSC versus freshly cultured MSCs. They found thawed MSCs have significantly reduced viability, substantially diminished capacity to inhibit in vitro T cell proliferation and decreased sensitivity to IFN- $\gamma$  compared to freshly isolated cells. The thawed cells showed a relative unresponsiveness to both pro inflammatory and antiinflammatory cytokine signals (21, 37). Fortunately, once these thawed cells are cultured for 24 hours, they do regain much of their immunomodulatory

capacities. Therefore, when cryopreserved cells are thawed, they must be cultured for a certain period of time before they are injected into patients. This finding clearly shows there is a difference between freshly cultured and freeze- thawed MSCs. It is important to consider this matter when treating patients with stem cells.

To address this issue, we sought to find a cell medium that would allow longer viability and functionality for freshly isolated and cultured MSC. Our medium of choice was human AB positive plasma. First, it has a human origin, which resolves the animal to human contamination issues. Second, we used AB positive plasma because it lacks blood type antibodies. Finally, plasma is the best medium for preserving the natural state of cells.

Our results show that MSCs isolated from bone marrow, adipose tissue and Wharton's jelly preserve their stemness in AB+ plasma medium whether they are kept in the fridge or at room temperature conditions. We believe MSCs isolated from adipose tissue preserve their proliferative ability better than MSC cells isolated from bone marrow and Wharton's jelly. This greater potency for expansion in culture conditions has been noted in other studies (38). The pattern of expansion for MSCs isolated from Bone marrow and Wharton's jelly is similar but shows a more pronounced drop in proliferation relative to adipose tissue isolated MSCs over time. AB+ plasma is a suitable candidate for keeping these cells for at least 4 days because it has no adverse effect on their biological characteristics and capabilities. This finding will prove invaluable for therapeutic use of these cells.

MSC cells must first be isolated from their source, cultured, expanded and harvested. If the patient and clinical conditions are favorable and transfusion is possible on the same day cells are harvested, they can be suspended in normal saline solution supplemented with 2.5% HSA and 1 U/ml heparin. As long as this solution is injected to the patient within 18 hours, the cells will retain their viability, biological characteristics and growth potential in the body. Although these cells maintain their growth and differentiation potential in normal saline conditions for 24 hours, considering the decrease in viability to below 80% (especially for cells isolated from bone marrow and Wharton's jelly), it is more reasonable to perform the transfusion within 18 hours. We also tested a few samples with suspension in 5% HSA. The results were similar to cells suspended in 2.5% HSA (Data not shown).

If it is not possible to inject cells within 24 hours after MSCs are harvested, the cells can be kept in AB+ human plasma for at least 4 days without compromising their biological characteristics and viability.

Our findings show that average viability and proliferative potential of cells in this medium remain above the acceptable range (viability of more than 80% and cell confluency above 80% within 20 days), however, some samples showed a decrease in these characteristics after 4 days. Our studies suggest MSCs isolated from adipose tissue can be kept in plasma for up to 7 days, however further validation is needed. Furthermore, although it is possible to keep these cells in plasma medium under fridge and room temperature conditions, we suggest cells be kept in fridge conditions (5+/- 3) because conditions are better maintained.

## **Suggestions for Further studies**

To further confirm the findings of this study, these MSCs can be evaluated for immunomodulatory capabilities and efficacy using in vitro tests on day 3 and day 5.

## **Conclusion**

This study evaluated the effect of time and temperature on MSCs stored in two mediums. These cells maintain their viability, growth and proliferative potential for 24 hours in normal saline. Whether kept in the refrigerator or at room temperature, cells kept in AB+ plasma maintained their characteristics for at least 4 days. Maintaining these cells in normal saline for 24 hours has been reported previously. This is the first study to evaluate ex-vivo expanded MSCs prepared for patient transfusion in AB+ plasma. We have demonstrated that cells maintain their normal capabilities and are in transfusion ready condition for up to 4 days when suspended in AB+ human plasma.

We are the first to report this novel method for maintaining freshly cultured MSCs in clinical grade injection ready state for more than 24 hours. The implications for this finding are astounding. This will make stem cell therapy much more accessible. It will decrease the cost considerably, since cryopreservation can be eliminated in many cases. It will also make it possible to establish a network for sharing and transferring injection ready MSCs globally.

## **Declarations**

### **Ethics approval and consent to participate**

The study design and the informed consents for adipose mesenchymal stem cells were approved by the Ethics committee of Baqiyatallah University of Medical Sciences (ethics code: IR.BMSU.REC.1393.32). For bone marrow MSC, approved by Tehran University of Medical Sciences (TUMS) and Shahid Beheshti University of Medical Sciences (ethics code: IR.SBMU.REC.1395.82). Whorton's jelly was obtained from Sina Cell. Sina cell provides questionnaires and informed consent papers for all donors at the time of collection.

### **Consent for Publication**

Informed consent was obtained from all donors at the time of collection (available upon request).

### **Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article. If further information is needed, the supplementary datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

## **Funding**

**Funding for this study was provided by SinaCell Company (Tehran, Iran).**

## **Authors' contributions**

Mandana Mohyeddin Bonab <sup>1</sup>: Project supervisor, experiment design, performed experimental procedure and cell culture, trouble shooting, manuscript preparation

Fatemeh Talebian<sup>2</sup>: Manuscript preparation and data analysis

Aida Borzabadi <sup>3</sup>: Statistical analysis, patient interaction

Vahideh Nasr<sup>3</sup>: Placenta cord procurement

Azam Abedi Kooshlshahi<sup>1</sup>: Performed experimental procedures and cell culture

Fahime Anisie<sup>1</sup>: Performed experimental procedures and cell culture

Ronaz Haghshenas<sup>1</sup>: Performed experimental procedures and cell culture

Bitā Shalbafan<sup>4</sup>: MSC procurement, patient interaction

Ehsan Janzamin<sup>5</sup>: flowcytometry analysis

Saeed Shahbeigi<sup>6</sup>: MSC procurement, patient interaction

## ***Acknowledgements***

The authors would like to thank Sinacell for providing funding for this study.

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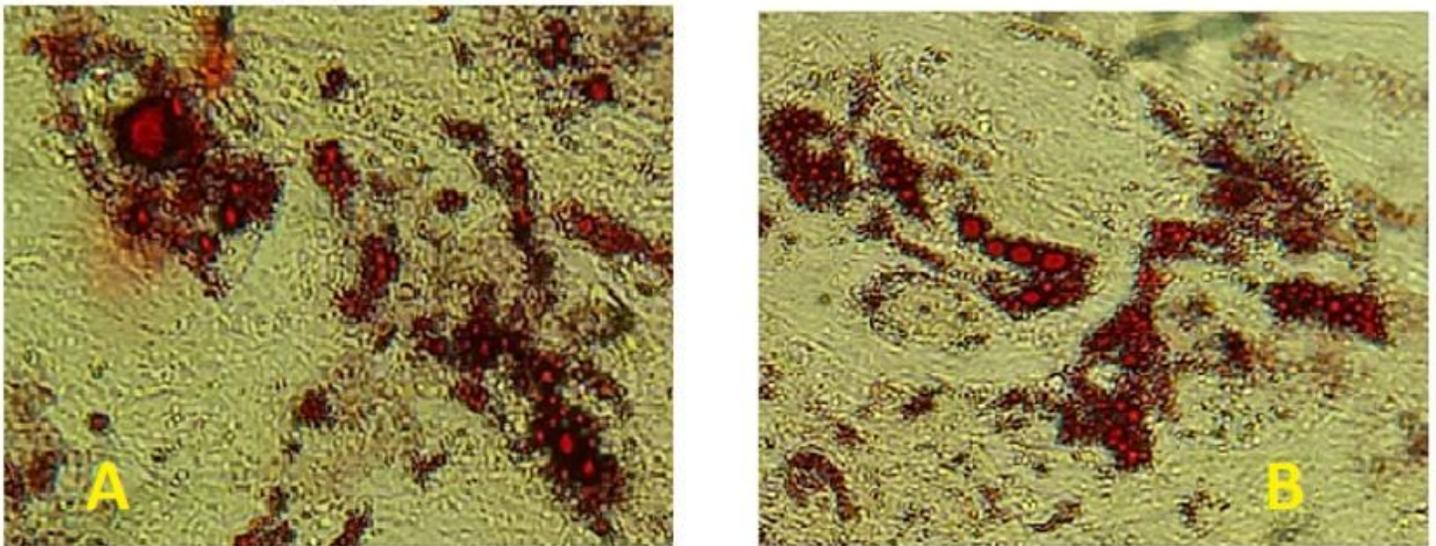
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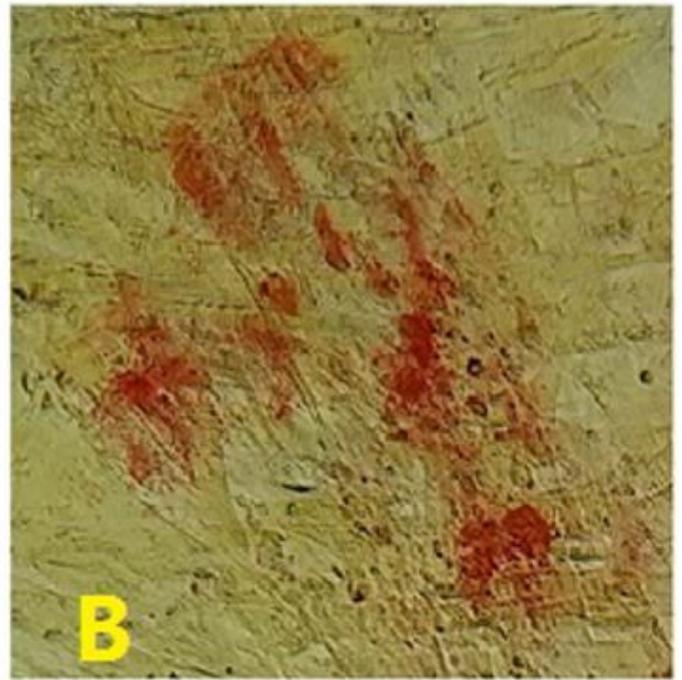
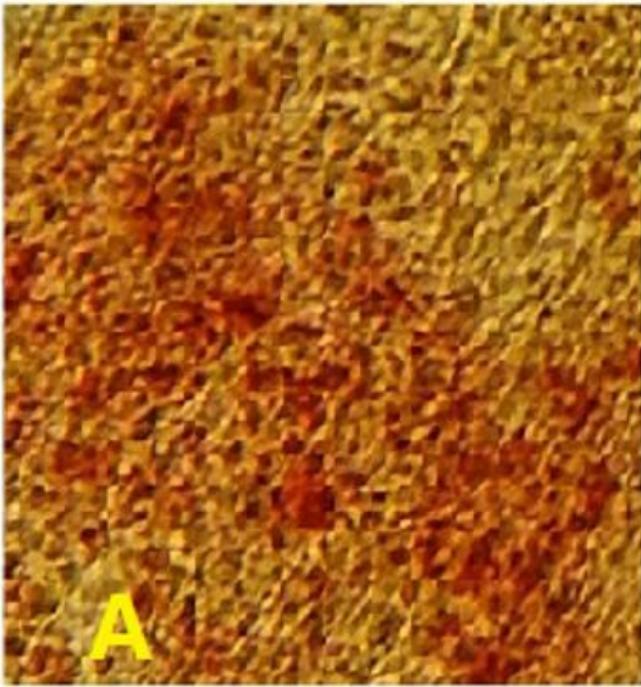
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## Figures



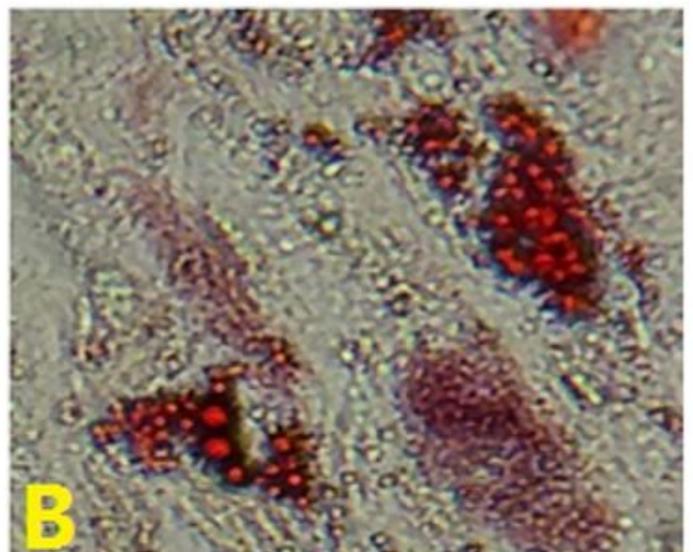
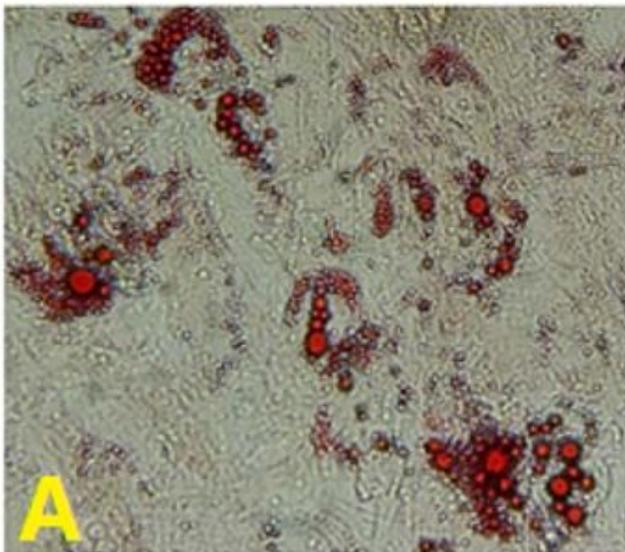
**Figure 1**

Oil red O staining showing fat drops in adipocytes of differentiated MSCs following 3 weeks of culture in adipocyte differentiation medium. Pictures taken after 18 hours of storage in normal saline. A: fridge temperature, B: room temperature.



**Figure 2**

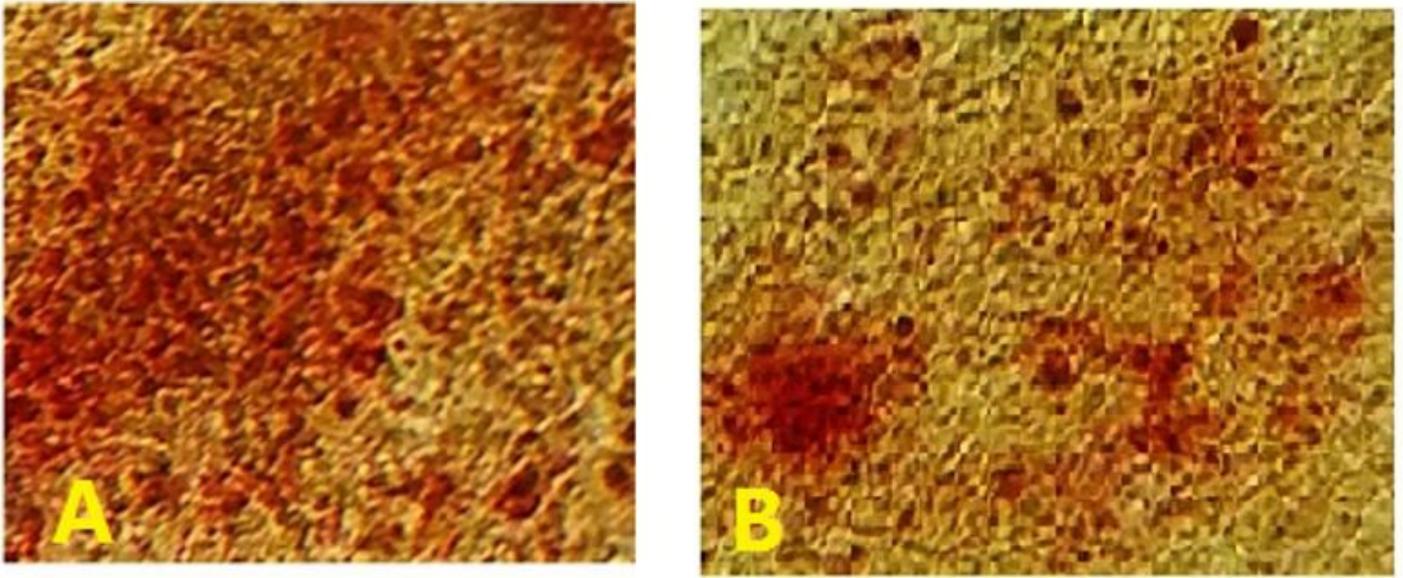
Alizarin red staining showing calcium deposits in osteocytes of differentiated MSCs following 3 weeks of culture in osteocyte differentiation medium. Pictures taken after 18 hours of storage in normal saline. A: Fridge, B: room temperature.



**Figure 3**

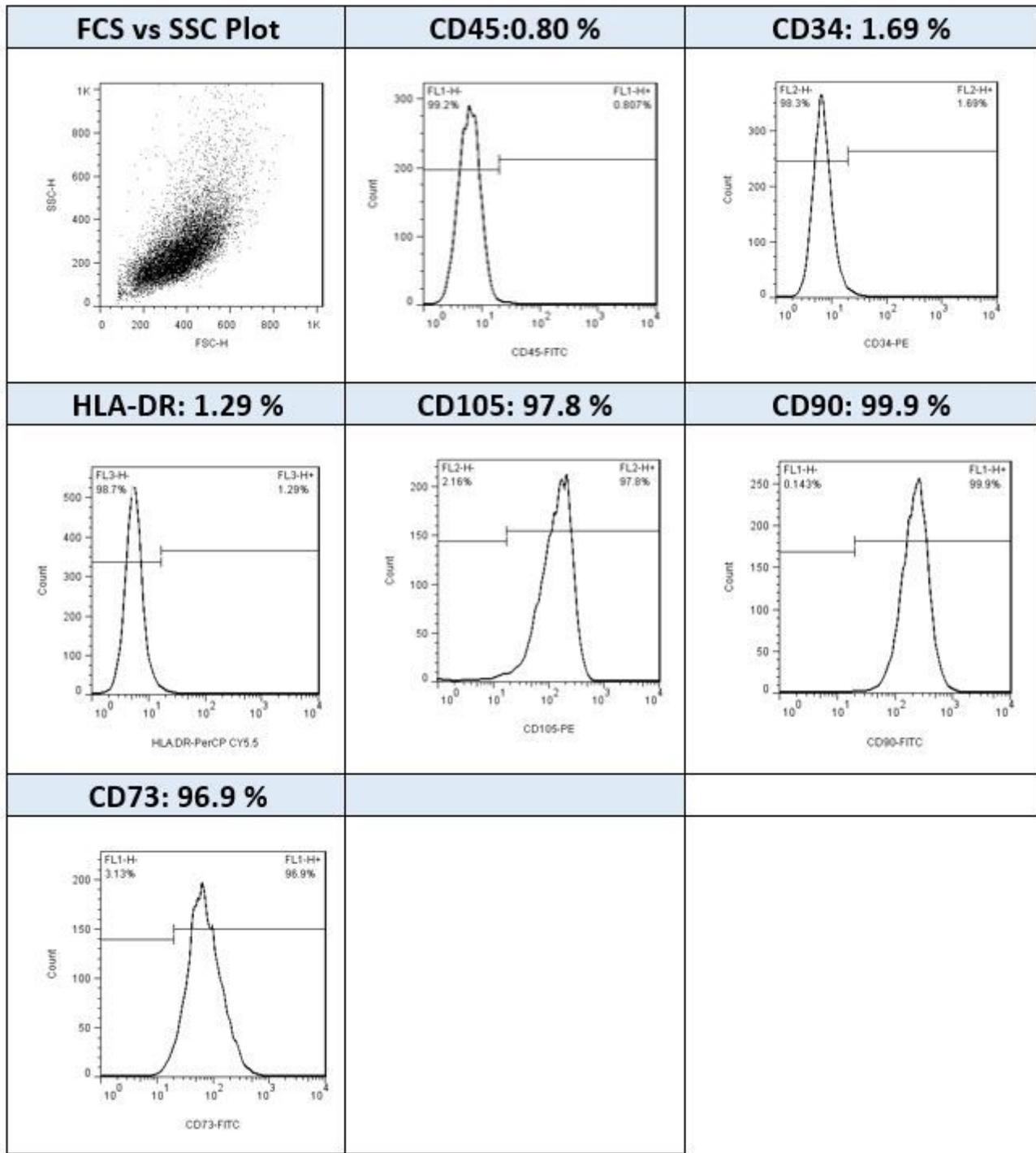
Oil red O staining showing fat drops in adipocytes of differentiated MSCs following 3 weeks culture in adipocyte differentiation medium. Pictures taken after storage for 3 days in AB+ human plasma. A: fridge

temperature, B room temperature.



**Figure 4**

Alizarin red staining showing calcium deposits in osteocytes of MSCs following 3 weeks culture in osteocyte differentiation medium. Picture taken after storage for 3 days in in AB+ human plasma. A: fridge, B: room temperature.



**Figure 5**

Example of MSC immunophenotyping after 3 days storage in AB+ human plasma.