

# Cultivation and Cryopreservation of Cord Tissue MSCs with Cord Blood AB Plasma

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Neonatal tissues, cord and placenta, are explored as alternate sources of mesenchymal stem cells (MSCs) for their therapeutic applications. Conventionally, MSCs isolated from cord tissues are maintained and propagated in FBS containing medium for promotion of growth and survival of cells. However, for therapeutic use, FBS use is not encouraged as it is of animal origin. Thus, there is a need for replacement of FBS by equally potent and clinically acceptable cost effective sources. The current study is designed to compare the effect of cord blood plasma (CBP) with MSC qualified FBS (M FBS) during culture and cryopreservation of MSCs. MSCs were isolated from cord and placenta and propagated in either M FBS or CBP. The efficiency of the cultures was analyzed by growth curve, morphology, phenotype and functionality. The cryo-protective role of the CBP was evaluated by using it in freezing medium of MSCs. Our data showed that CBP is equivalent to M FBS for culturing placental MSCs with respect to the phenotype, proliferation rate and differentiation to various lineages. However, cord MSCs displayed slow growth rate and reduction in surface expression of CD105 marker in CBP, whereas, the other parameters were comparable. Freezing of MSCs with CBP resulted in reduction of the late apoptotic and necrotic population. Thus, CBP imparts superior protection against cryogenic insults, and appears to be a valuable substitute to M FBS for cultivation and freezing of MSCs.

## INTRODUCTION

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells with multi-differentiation potential. MSCs have gained a substantial therapeutic value because of their plasticity and immune suppressive characteristics (Knaan-Shanzer, 2014). MSCs are primarily isolated from bone marrow (BM) aspirates. Owing to the invasive procedure and limitation in amount of sample procured, alternate sources such as umbilical cord,

placenta, endometrial polyps, menstrual blood, adipose tissue, are being harnessed to obtain MSCs (Moroni and Fornasari, 2013).

Cord/placenta derived MSCs need to be cryopreserved so that they can be revived and used in clinics when required. Generally, the protocols use specialized FBS like mesen FBS (M FBS) for culturing and freezing MSCs (Ng *et al.*, 2008; 2014). However, clinical transplantation protocols demand replacement of xenogeneic products to avoid adverse

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immunological reactions and possible transmission of infectious agents (Ma *et al.*, 2012). Several reports indicate use of serum free media for culture and expansion of MSCs (Al-Saqi *et al.*, 2014). However, the protocols are expensive and not as effective as serum containing media. Thus, there is a need for substitutes for xenogeneic sera including autologous serum (Wang *et al.*, 2012), autologous plasma (Lin *et al.*, 2005), PRP (Pham *et al.*, 2014), platelet lysates (Iudicone *et al.*, 2014). However, availability of human blood, plasma, platelets from blood banks is difficult, expensive and considered unethical. Cord blood banking opened a new avenue for regenerative medicine. During banking of the samples, the RBCs are separated to obtain the mononuclear cells (MNCs) and hematopoietic stem cells (HSCs). At this time if the plasma is collected and stored separately, it can be utilized for culturing cord/placenta MSCs and thus the fractionated products of cord blood can be put to use in the clinics.

In the current article, cord blood derived AB positive plasma (CBP) has been used for both culture and freezing of MSCs isolated from cord (C MSCs) and placenta (P MSCs) and its efficiency compared with M FBS. Our data indicates that CBP can serve as a valuable substitute to FBS, and be acceptable in the clinics.

## **MATERIALS AND METHODS**

### **Isolation of MSCs**

Umbilical cord and placenta were collected from full term deliveries from clinics and from

independent donors. The collection was performed with informed consent as per the protocols approved by the Institutional Ethics Committee (IEC), NCCS, Pune (11<sup>th</sup> IEC/IC-SCRT of NCCS held on 20<sup>th</sup> Jan 2012). Umbilical cord and a section from the central part of the placenta were used to isolate the MSCs. The tissues were washed in Iscove's modified Dulbecco's medium (IMDM) (Sigma Aldrich, St Louis, USA) and chopped into pieces and then subjected to enzymatic digestion with 0.25% trypsin for 30 min. Single cell suspension was obtained by passing the homogenate through sterile muslin cloth. The cells were washed with medium and suspended in complete growth medium RPMI 1640 (Sigma Aldrich, St Louis, USA) with 20% M FBS (Gibco, Invitrogen, Grand Island, USA) and seeded in 75 cm<sup>2</sup> tissue culture flasks (Falcon, Beckton Dickinson, San Jose, CA, USA). Non-adherent cells were removed after 72 h. Cultures were re-fed with fresh medium after every 72 h for 10–20 days. The cultures were maintained by passaging them after reaching 70–80% confluency, for 6–7 passages. The cells between passage numbers 3–6 were used for the experiments after confirming the phenotypic signature of the MSCs. Adaptation of cultures to CBP was initiated in passage 2, described below.

### **Collection of AB positive plasma from cord blood**

Cord blood samples were collected from local hospitals with the compliance of the institutional review board (IEC, NCCS).

Blood grouping was carried out with ERYSCREEN reagent (Tulip Diagnostic, Mumbai, India). From the AB positive samples, mononuclear cells and plasma were simultaneously isolated by a single step method using Ficoll Hypaque (Sigma Aldrich, St. Louis, MO, USA) density gradient separation (Density 1.077 g/ml). The separated plasma was collected and complement inactivation was carried out at 56 °C for 30 min. After removing aliquots for sterility checking, the plasma was stored frozen at -20 °C and further used for maintenance of MSCs. As AB plasma is only used for the experimental purpose the levels of endotoxin were not checked. The MNCs obtained during plasma collection were utilized for the isolation of hematopoietic stem cells. AB positive plasma from three independent cord blood units were used for the experiments.

#### **Culture adaptation and propagation of MSCs to CBP**

For adaptation to CBP we used MSCs from passage 2 (60–70% confluent), washed 3–4 times with PBS before changing the plasma containing medium. C MSCs (n = 3) and P MSCs (n = 3) in passage 3 were harvested and divided in two sets, and cultured in RPMI + 20% M FBS and RPMI + 20% CBP, with equal seeding density in T25 cm<sup>2</sup> flasks. After the cells reached confluence they were harvested and used for subsequent experiments. Traces of FBS after CBP adaptation were not checked.

#### **Growth kinetic study**

1 × 10<sup>3</sup> cells were seeded in 96-well plates in RPMI + 20% M FBS/CBP containing medium. Proliferation was assessed by MTT assay (Sigma Aldrich, St Louis, USA). The cultures were followed for 144 h and absorbance measured at 570 nm.

#### **Characterization of MSCs by flow cytometry**

MSCs expanded from cord or placental tissue were characterized by the following antibodies – CD105-PE, CD73-APC, CD166-PE, CD90-APC, CD34-PE (Beckton Dickinson Pharmingen, San Jose, California, USA) and CD45-APC (eBiosciences, San Diego, USA). Isotype matched antibodies were kept as controls. The fluorescent labeled cells were acquired on fluorescence activated cell sorter (FACS) Canto II (Beckton Dickinson, San Jose, CA USA). An acquisition of 10,000 events was done and data analyzed by FACS DIVA, version 5.0.

#### **Colony forming unit – Fibroblast assay (CFU-F)**

5 × 10<sup>3</sup> MSCs were seeded in 60 mm dish with RPMI + 20% M FBS/CBP containing medium and incubated at 37 °C, 5% CO<sub>2</sub> humidified environment for 7–10 days. The non-adherent cells were removed, the monolayer was washed with PBS and fixed using 100% methanol (Fischer Scientific, Mumbai, India) for 10 min. The cells were stained with 0.1% crystal violet solution for 10 min. Clones of > 50 cells were scored as CFU-F. Staining was done in triplicates for each sample.

### **Differentiation to osteoblasts and adipocytes**

$1 \times 10^4$  MSCs were seeded in 24-well plates (Falcon, Becton Dickinson, San Jose, USA) and grown to 60–70% confluency. The cells were then subjected to osteogenic and adipogenic differentiation for 15–18 days using kit (STEMPRO® Osteogenesis/Adipogenesis Differentiation Kit, Invitrogen, USA). The osteogenic and adipogenic differentiation was confirmed by performing Alizarin Red S (Sigma Aldrich, St Louis, USA) staining for calcium deposits and intracellular lipid droplets staining with Oil red O dye (Sigma Aldrich, St Louis, USA), respectively.

### **Cryopreservation of MSCs**

$1 \times 10^6$  MSCs were cryopreserved in conventional freezing medium (RPMI + 20% M FBS/cord blood plasma) containing 10% DMSO by portable programmable freezer (Freeze Control, Victoria, Australia) at the controlled cooling rate of 1 °C/min to –40 °C followed by 10 °C/min to –90 °C and then kept in liquid nitrogen. The cells were thawed by rapidly immersing the vials in a water bath at 37 °C. The viability of the cells was assessed by trypan blue dye exclusion method. Further, apoptotic profiling was carried out by performing Annexin V and PI staining (Becton Dickinson Biosciences, San Jose, CA, USA) as per manufacture's instructions and analyzed by flow cytometry.

### **Statistical analysis**

The differences between growth of C MSCs in FBS and C MSCs in CBP ( $n = 3$ ), and between growth of PMSCs in FBS and P MSCs in CBP ( $n = 3$ ) were compared by a one way repeated measure analysis of variance using the software SIGMA STAT (Jandel Scientific Corporation, San Rafael, CA, USA). The values were plotted as mean  $\pm$  standard deviation. Probability values of  $p \leq 0.05$  were considered statistically significant.

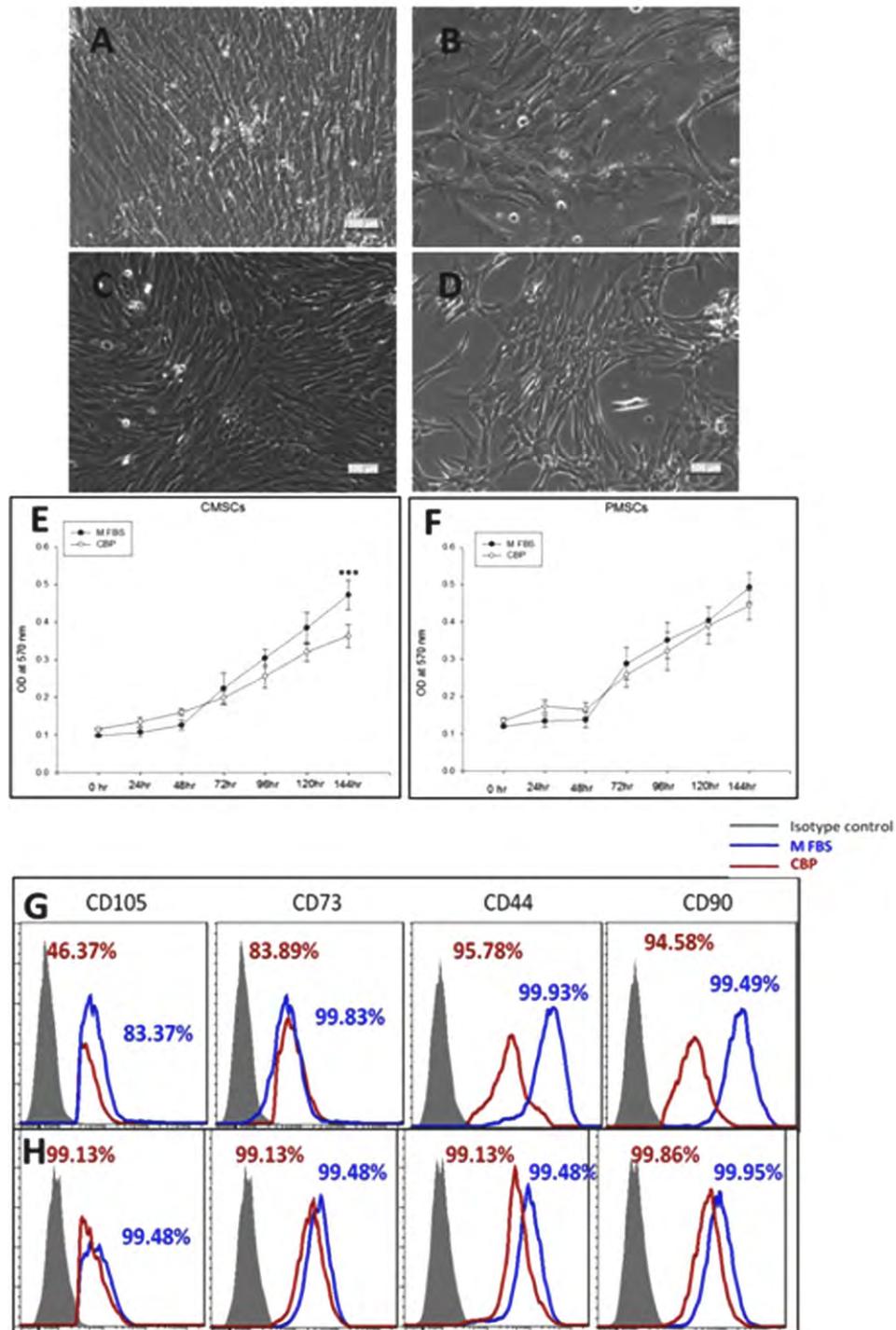
## **RESULTS**

### **Morphological differences in MSCs cultured in M FBS vs CBP**

MSCs cultured in M FBS and CBP showed typical fibroblast morphology (Fig. 1A–D). However, C MSC and P MSC growth in CBP was aberrant, with persistent clusters observed in the cultures. The clusters were more prominent in C MSCs (Fig. 1B) as compared to P MSCs (Fig. 1D) indicating a delayed growth of C MSCs as compared to P MSCs. This pattern of growth in C MSCs may be attributed to low seeding density as this trend was reversed by increasing seeding densities.

### **C MSCs grown in FBS show higher proliferation rate**

Growth kinetics of the MSCs in M FBS vs CBP were investigated to check whether the differences in morphological appearances of the cultures were reflected in the proliferation rates. The C MSCs with M FBS showed higher growth rates as compared to those grown in



**Figure 1: Morphology, growth kinetics and phenotype of MSCs grown with M FBS and CBP:** Phase contrast images of C MSCs and P MSCs grown in M FBS (A and C, respectively) and CBP (B and D, respectively). Comparison of growth kinetics of C MSCs (E) and P MSCs (F) in M FBS and CBP. Surface marker analysis by flow cytometry of MSCs grown in either M FBS or CBP in both C MSCs (G) and P MSCs (H).

CBP and the difference was significant at day six (Fig. 1E). However, P MSCs showed more or less similar proliferation patterns with FBS/plasma (Fig. 1F).

**P MSCs grown in M FBS/CBP show equivalent level of expression of surface markers**

We next proceeded to characterize the MSCs grown with FBS/plasma for the expression of surface markers. Significant differences were not observed with P MSCs/C MSCs in both media for surface marker expression of CD73, CD90 and CD44. However, C MSCs showed 50% less expression of CD105 when grown in AB plasma. Histogram overlays for a representative sample for using a panel of antibodies are depicted in Figs. 1G and H for C MSCs and P MSCs, respectively. The MFI levels were increased in C MSCs with M FBS as compared to those seen in CBP.

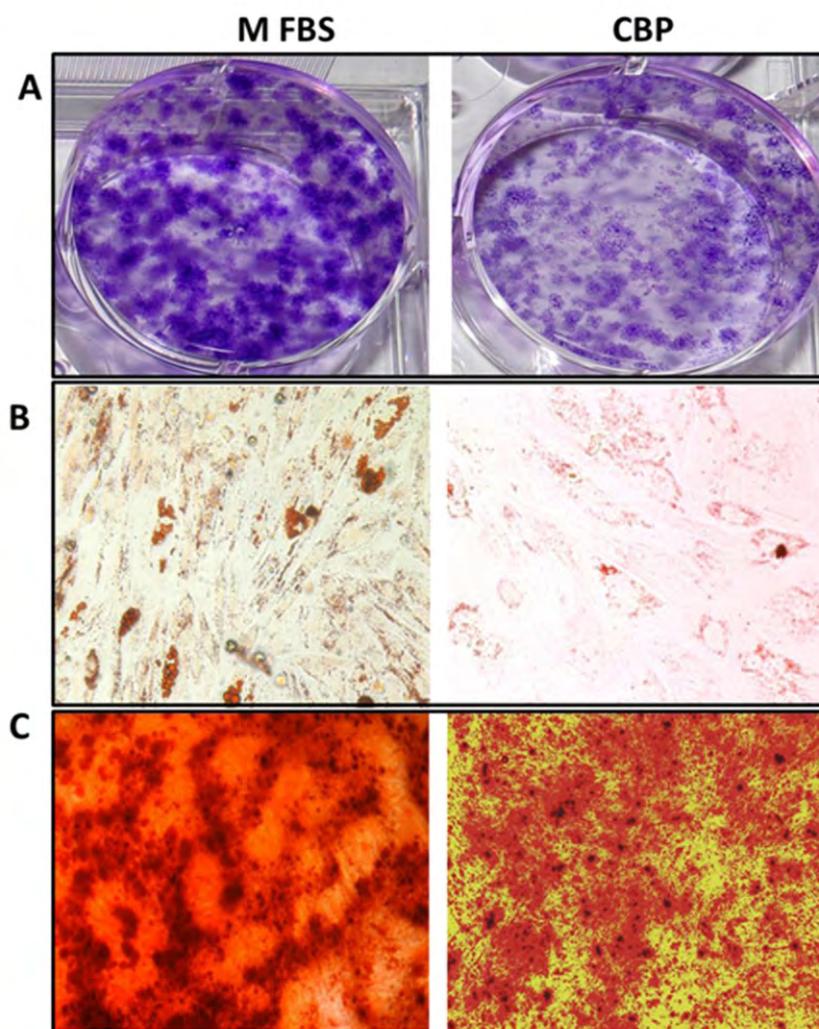
**MSCs cultured in CBP have comparable CFU-F potential and differentiation ability**

The clonogenicity of MSCs was assessed by CFU-F assay. MSCs in CBP showed  $120 \pm 5$  CFU-F and in M FBS showed  $150 \pm 10$  CFU-F per  $5 \times 10^3$  cells indicating comparable potential in both media. Differentiation of MSCs to adipocytes and osteoblasts was similar in M FBS and CBP containing media. Images of CFU-F (Fig. 2A) and differentiation to adipocytes (Fig. 2B) and osteoblasts (Fig. 2C) of representative MSCs in M FBS and CBP are depicted.

**Cord plasma is a useful substitute to FBS for cryopreservation of MSCs**

The cells are subjected to stress during cryopreservation and may be enhanced if the culture medium and freezing media are different. Hence, C and P MSCs grown with M FBS and CBP were cryopreserved in the respective media containing 10% DMSO and 20% FBS/Plasma. The viability post revival by trypan blue dye exclusion test ranged from 75–80% in all the sets. Cryopreserved MSCs were revived and assessed for retention of the cell surface markers. Post thaw phenotypic analyses did not reveal any significant difference in expression of markers like CD44, CD73, and CD90 in the two sets, except with the CD105 expression in C MSCs (Table 1).

The revived MSCs from the two sources frozen with two different media were subjected to Annexin V–PI staining and analyzed on flow cytometry for the percentage of viable, apoptotic and necrotic cells. C MSCs frozen with FBS showed less ( $48.00 \pm 6.23\%$ ) viable cells as compared to CBP ( $61.10 \pm 9.93\%$ ), whereas P MSCs showed similar number of viable cells ( $58.57 \pm 10.93\%$ ) in the two sets. Lesser percentage ( $9.27 \pm 3.51\%$  and  $3.10 \pm 1.37\%$ ) of early apoptotic cells and more ( $24.87 \pm 9.05\%$  and  $23.65 \pm 7.27\%$ ) late apoptotic and necrotic cells ( $17.8 \pm 6.38\%$  and  $14.72 \pm 2.28\%$ ) were observed in both C and P MSCs frozen in M FBS, respectively. On contrary, the percentage of late apoptotic and necrotic cells were significantly reduced (less than 10%) for both



**Figure 2: Microphotograph depicting *in vitro* functionality of MSCs and CBP:** (A) Distribution of colonies of MSCs in CFU-F cultures grown in M FBS and CBP. MSCs cultured in M FBS and CBP subjected to (B) adipogenic and (C) osteogenic differentiation and stained with oil red O and Alizarin red S, respectively.

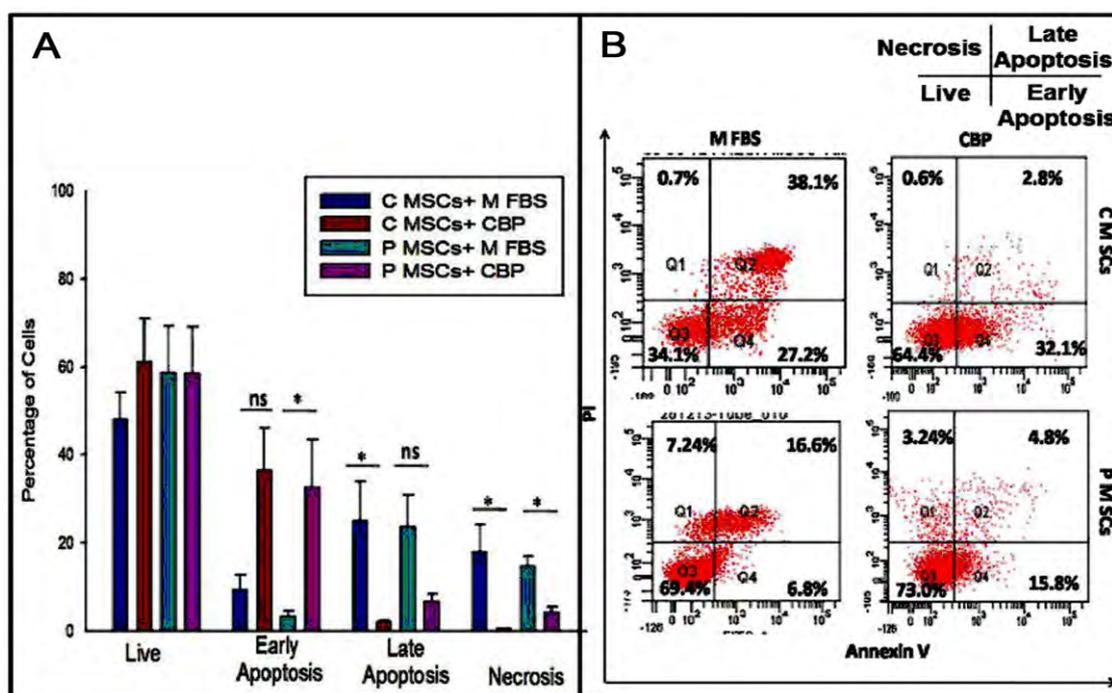
C and P MSCs frozen with CBP with a relatively higher percentage of early apoptotic population ( $36.33 \pm 9.80\%$  and  $32.60 \pm 10.89\%$ ) (Fig 3A). A representative FACS profile is depicted in Fig 3B. This data suggests that CBP is more efficient than M FBS in protecting the MSCs from cryo injuries.

## DISCUSSION

BM-MSCs are difficult to obtain and their quality deteriorates with age. So cord tissue derived MSCs are an alternative for cell based therapies (Moroni and Fornasari, 2013). Ready availability and ontogenetically conserved nature of the umbilical cord tissues prompted us to assess cord and placenta for

**Table 1:** Post thaw phenotypic analysis for expression of markers in C MSCs and P MSCs (\*\* $P < 0.01$ ).

| Marker | C MSCs        |                | P MSCs       |               |
|--------|---------------|----------------|--------------|---------------|
|        | M FBS         | CBP            | M FBS        | CBP           |
| CD105  | 83.03 ± 12.77 | 35.06 ± 7.52** | 75.5 ± 24.09 | 76.06 ± 23.89 |
| CD44   | 97.23 ± 1.49  | 99.8 ± 0.1     | 97.93 ± 0.49 | 99.93 ± 0.05  |
| CD73   | 97.8 ± 1.24   | 95.86 ± 1.02   | 98.33 ± 0.58 | 97.83 ± 2.54  |
| CD90   | 97.63 ± 1     | 99.7 ± 0.36    | 95.87 ± 2.45 | 99.46 ± 0.75  |
| CD34   | 0.43 ± 1.02   | 0.4 ± 0.26     | 0.57 ± 1.46  | 0.76 ± 0.25   |
| CD45   | 0.67 ± 1.06   | 2.70 ± 0.69    | -0.43 ± 0.4  | 3.2 ± 0.3     |

**Figure 3: Revival of MSCs froen in M FBS/CBP:** (A) Annexin V staining shows significant decrease in the late apoptotic and increase in the early apoptotic population when MSCs were frozen with CBP; (B) Representative FACS profiles of Annexin V and PI staining for MSCs frozen/revived with M FBS and CBP, respectively. \* $P < 0.05$ .

isolation of MSCs. No significant difference was observed in major characteristics including morphology and phenotype of MSCs obtained from the two sources (data not shown). However, inherent differences in these populations need to be extensively investigated.

MSCs are generally propagated in serum containing media. Due to the xenogeneic nature of FBS, an elevated risk of transmitting infectious agents and adverse immunological reactions is a distinct possibility (Ng *et al.*, 2008; 2014). In the current study we have attempted to culture and cryopreserve

clinically compliant MSCs by replacing M FBS with CBP in the media. We initially isolated the MSCs in the FBS containing medium as FBS has cell stimulatory and survival promoting factors essential for the initial survival. The MSC colonies were adapted to CBP. The aim of the study was to culture and adapt the MSCs to AB positive CBP. Due to the availability of cord blood, as often it is discarded, CBP is a cost effective substitute for FBS. We report the use of CBP for cultivation and cryopreservation of MSCs isolated from both cord and placenta. We preferred plasma over serum to curtail loss of precious stem cells within the clotted fraction while collecting serum. Autologous plasma from human source has been studied in the context of BM-MSCs. As we are studying cord/placenta derived MSCs, CBP was preferred to peripheral blood derived plasma as a suitable substitute for FBS which contributes to xenogeneic proteins. Considering the fact that under *in vivo* conditions placenta, cord and cord blood demonstrated similar interactions, the cultures may adapt to CBP. AB positive plasma was the choice due to absence of antibodies for either antigen which may influence the growth and development of MSCs.

Our data demonstrated CBP as an effective substitute to M FBS as characterized by the growth pattern, phenotypic signature, clonogenic ability and differentiation capabilities of both C MSCs and P MSCs. However, the observed lag in the growth kinetics for C MSCs in CBP may be attributed

to the delay in the adaptation to the serum replacement. The lag period can be shortened by increasing the seeding densities during the initial passages, whereas P MSCs showed comparable growth in both media. The immunophenotype of the both MSCs was similar with the exception of reduced CD105 expression by C MSCs in CBP. Mark *et al.* (2013) have reported decreased CD105 expression in serum free medium as compared to serum containing media. Our data showed comparable CD105 expression for PMSCs which may be attributed to a more adaptable nature of the cells. A functional assay for MSCs is the multi-lineage differentiation capacity of the cells. Our data showed that MSC adaptation to CBP retained the multi-lineage differentiation capacity with minor variations in the differentiation exhibited by MSCs grown in FBS vs CBP. It has been reported that variations in culture conditions influence the differentiation potential of MSCs (Al-Saqi *et al.*, 2014). Perhaps the observed differences may be attributed to a proliferation delay in CBP containing cultures. Cryopreservation of MSCs in chemically defined media is essential for application as ready to use off the shelf cell products in therapeutics. Roy *et al.* (2014) replaced serum by sucrose during cryopreservation of MSCs and reported that the cells were compromised. We have demonstrated CBP as a better cryoprotectant over conventional FBS in the freezing medium. Substitution of FBS with CBP in the freezing medium in our studies had no adverse effect on viability of the cells as

detected by both trypan blue dye exclusion and Annexin V-PI staining. Interestingly, MSCs frozen with CBP exhibited enhanced protection from cryo-injury, as there was a significant reduction in the late apoptotic and necrotic population as opposed to MSCs frozen with FBS. Though we observed an increase in the early apoptotic population (Annexin V<sup>+</sup> PI<sup>-</sup>) for CBP set as compared to FBS set, this population can be rescued back to the Annexin V<sup>-</sup> PI<sup>-</sup> double negative phenotype (viable) by continual maintenance at 37 °C (Chinnadurai *et al.*, 2014; Geske *et al.*, 2001).

Recently, Ng *et al.* (2014) described fetal extracellular matrix proteins for culturing adult MSCs, thus providing natural biological supports compared to synthetic polymers. With an increasing trend towards use of natural non-xenogenic substitutes for culture, cryopreservation and expansion of MSCs, CBP is a potential, economical and valuable substitute for FBS in culture and freezing media for MSCs. However, adaptation of the cultures for a few passages to improve growth

patterns and expression of surface molecules while retaining their genetic stability needs to be explored. Further, prior to use of CBP adapted MSCs presence of endotoxins should to be checked.

Our data suggests that CBP may be a relevant substitute for FBS in clinical applications.

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#### CONFLICT OF INTEREST

The authors claim no conflict of interest.

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