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Factors Affecting Growth and Proliferation Mesenchymal Stem Cells (MSCs) Derived from Adult Bone Marrow

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Abstract—*In vitro manipulation of various biological, physical and chemical factors influences growth, propagation and proliferation of in vitro cultured mesenchymal stem cells (MSCs) derived from adult bone marrow. Here, different factors were investigated that are responsible for growth, propagation and proliferation. Among the investigated seeding density (1×10^4 cells/ml, 1×10^5 cells/ml and 1×10^6 cells/ml), the seeding rate 1×10^5 cells/ml generate highest number of MSC-like colonies (130 ± 3. 48) in comparison to 1×10^4 cells/ml (9 ± 0.7) and 1×10^6 cells/ml (31± 1.47) after culturing for same interval (7th passage). It is also interesting to note that the number of colony formation declined after 4th passage in case of 1×10^4 cells/ml and 1×10^6 cells/ml whereas there was a pragmatic increase in number of colony formation even observed after 4th passage at 1×10^5 cells/ml. Among the studied temperature the highest cell proliferation observed (8900991.25 ± 4.76) obtained at 37°C in comparison to (5065207.5 ± 1.84) at 33°C, (1001989 ± 2.16) at 29°C, (500096.75 ±1.49) at 25°C, (100054 ± 2.97) at 21°C and (90003 ± 1.47) at 41°C. In this study, we examined the performances of six different concentration of fetal bovine serum (FBS) 1%, 3%, 5%, 7%, 10% and 15% on cell proliferation. Our results indicate that the rate of MSCs proliferation increased as the concentration of FBS increases from 1% to 10% and maximum cell proliferation obtain at 10% FBS (25670898±2.94). Here, the addition of granulocyte-macrophage colony stimulating factor (GM-CSF) acts as a strong enhancer of MSC-like colony formation. However, highest number of colony formation (150 ±1.82) occurs at 10 ng/ml of GM-CSF. In this report we define the "prime inductive factors" that chiefly responsible for successful isolation and expansion of human MSCs in higher number for successive stem cell based therapeutic approaches.*

Keywords: *Bone marrow, mesenchymal stem cells, factors, growth, expansion, therapeutics.*

1. INTRODUCTION

Mesenchymal stem cells or marrow stromal cells (MSCs) are atypical multipotent stem cell population, resides mainly in bone marrow (BM) and other tissue location. These are efficient cellular source currently being used in used in different aspects of regenerative medicine and cell-based therapies. However, surplus number of *in vitro* cultured MSCs is required for fulfilling the desired therapeutic interventions [1]. The most preferred approach to produce higher number of MSCs is to provide an optimized *in vitro* condition that mimics the *in vivo* environment. The *in vitro* culture environment mediated by several intrinsic and extrinsic factors regulated by different biophysical signals, physicochemical issues and biochemical cues [2]. So by manipulating, mediating and maintaining different parameters support growth and proliferation of *in vitro* cultured MSCs.

Therefore, in this study we attempted to study different biological, physical and chemical factors that regulate efficient MSCs production in adequate number for its successful application in cell-based therapies and translation research.

2. MATERIALS AND METHODS

2.1. Collection of bone marrow

Bone marrow cells were aspirated from healthy donors (n = 4, 2 men and 2 woman) between age group 23–50 year with a mean body mass index (BMI) of 23.86 ± 0.31 kg/m^2. Written informed consent was obtained prior to sample collection in accordance with the requirements of the ethical committee. Five to twenty ml of the sample was aspirated from the iliac crest region of four subjects. The collected samples were processed within two hour of collection.

2.2. Cell isolation and culture

Aspirated marrow were subjected to density gradient centrifugation after layering on Ficoll Paque™ (Himedia, India). The buffy coat was containing mononuclear Cell (MNC) was obtained after centrifugation for 20 minutes at 1800 rpm. The MNC fraction was collected and washed twice with sterile PBS. First the MNC suspension was seeded at a concentration of 5 x 10 ^{5} cells/ml in 0.1% gelatin coated T-25 flasks (Nunc, Roskilde, Denmark) and culture alongwith conditioned growth medium composed of Roswell Park Memorial Institute (RPMI) 1640 medium, 5% fetal bovine

serum (FBS), 100 IU ml^{-1} penicillin and 0·1 mg ml^{-1} streptomycin were added (all from Gibco-Invitrogen, USA). The cultures were incubated at 37ºC with 5% CO_2 (Contherm, Hult City, New Zealand) and observed regularly. The medium was change at every three days interval. After the attainment of 80% confluency these bone marrow derived MSCs (BM-MSCs) were harvested by using standard trypsinization procedure. Cell density was measured using haemocytometer. The cells were subjected to different temperature, various concentration of FBS, different concentration of GM-CSF to observe viability, growth and expansion at various interval.

2.3. Effect of seeding density on cell growth and propagation

The isolated cells were distributed at different seeding density 1×10^4 cells/ml, 1×10^5 cells/ml and 1×10^6 cells/ml into the wells of 0.1% gelatin coated six-well plates containing condition medium. Cultures were examined daily for the appearance of stromal colonies. Passaging was done at 5–7 day intervals by trypsinization. Prior to passaging the growth of cells was estimated by counting the number of MSC-like cell colonies formed at each seeding density under phase contrast microscope (Olympus, USA).

2.4. Effect of temperature on cell proliferation

Approximately, a total of 1×10^5 cells/ml (best determined from previous experiment) were seeded into different six-well plates and incubated at different temperature such as 21˚C, 25˚C, 29˚C, 33˚C, 37˚C and 41˚C. After three days of incubation, the cell density was measured using haemocytometer.

2.5. Effect of FBS concentration on cell proliferation

The effect of FBS concentration on cell proliferation was measured after 10th passage. A total of 1×10^5 cells/ml were first seeded into different six well plates supplemented with growth medium containing six different FBS concentration (1%, 3%, 5%, 7%, 10% and 15%). and incubated at 37˚C (optimum). Every other day, duplicate plates from each specific FBS concentration were washed twice with PBS (pH= 7.4). Then 0.25 % trypsin/ EDTA solution (Himedia) was added at room temperature for 1-2 minutes. The cells were dissociated when the cells were rounded up, cell density was measured using haemocytometer. The estimated cell number was expressed as cells/ml. The experiments were conducted continuously for 15 days.

2.6. Effect of granulocyte macrophage-colony stimulatory factor (GM-CSF) on MSC-like colony formation

A total of 1×10^5 cells/ml were seeded different six well plates containing growth medium RPM, I, 10% FBS (optimum), 100 IU ml^{-1} penicillin and 0·1 mg ml^{-1} streptomycin and incubated at 37˚ C. After 16 to 24 h the old medium was removed and replaced with medium containing different concentration of GM-CSF (5 ng/ml, 10 ng/ml, 15 ng/ml and 20 ng/ml). After five-seven days the number of colonies developed was estimated at each GM-CSF concentration.

2. 7. Growth kinetics study

2.7.1. Extrapolation of growth curves

The cultures were grown in an optimized environment. Initially the MNCs were seeded at the rate 1×10^5 cells/ml in the medium containing RPMI, 10% FBS and 10 ng/ml GM-CSF100 IU ml^{-1} penicillin and 0·1 mg ml^{-1} streptomycin, incubated at 37˚C. In order to plot the growth curves, BM-MSCs of passage 2 (P2), passage 6 (P6) and passage 10 (P10) were cultured continuously for 7 days. The cells from respective passage were harvested every day and cell density was determined microscopically for 0-7 days.

2.7.2. Calculation of population doubling time (PDT)

PDT was determined for P2, P6 and P10 cells as per formula

PDT=culture time (CT) / population doubling (PD).

PD = log (N / N0)/log2,) (N= cell number at culture end and N0 the number of the cells at culture initiation).

3. STATISTICAL ANALYSIS

All the experiments were conducted four times and data were expressed as mean ± standard error (mean ± S.E). Statistical analysis was performed using by using Statistical Package for Social Sciences (SPSS) version 20 under windows. Data were analyzed by one-way ANOVA to test the significant difference between the groups. Further, a value of $p \leq 0.05$ was considered to be statistically significant at 5 % level.

4. RESULTS

To define the actual cell concentration of MSCs that is responsible for growth and propagation first the MNCs were seeded at density 5×10^5 cells/ml in Nunc flasks (Figure 1 A). After seeding, these cells were attached to the plastic culture flasks and formed adherent monolayer within 7-10 days (Figure 1 B). After 10 days of plating, a mixture of cell types was observed with various morphologies growing in monolayers in some areas. The multi-cellular macrostructures protruding up into the medium termed as colonies were seen in the culture flasks after 14 days (Figure 1 C).

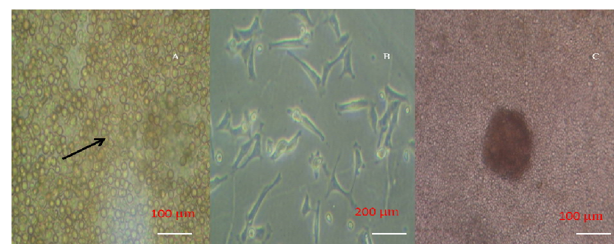

Figure 1 A. Isolated monocytes from BM B. Attached MSCs at p0 level. C. Round shaped MSC-like cell colony developed after 14 days of culture.

4.1. Evaluation of optimum seeding density

Subsequently, these adherent cells (passage 0 cells [P0]), which represent a MSC-enriched population, were trypsinized, counted and distributed to three different cell concentration as described in Materials and Methods, The growth of cells was determined by counting the number of MSC-like colonies formed at different passage level. According to our result among the tested seeding density 1×10^4 cells/ml, 1×10^5 cells/ml and 1×10^6 cells/ml, the formation of maximum number of MSC-like colonies occur at 1×10^5 cells/ml (130 ± 3.3) compared to 1×10^4 cells/ml (11±1.47) and 1×10^6 cells/ml (31±2.05) at passage 7. It is interested to record that that the number of colony formation declined after 4th passage in case of 1×10^4 cells/ml and 1×10^6 cells/ml whereas there was a pragmatic increase in number of colony formation even observed after 4th passage at 1×10^5 cells/ml (Figure 2).

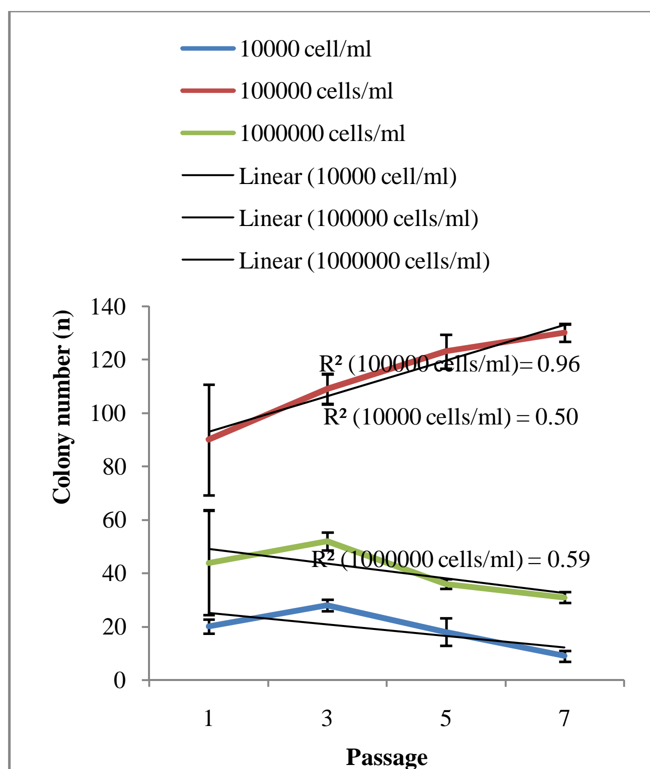

Figure 2 Estimation of MSC-like colonies generated at various passage level after seeding 1×10^4 cells/ml, 1×10^5 cells/ml and 1×10^6 cells/ml.

Additionally, it was recorded that there exist a strong relationship between seeding density and number of MSC-like colonies generated. It is evident that a stronger relationship yields a better result. It is predicted from the value of co-efficient of determination (R^2), 0.59 for 1×10^4 cells/ml, 0.96 for 1×10^5 cells/ml and 0.5 for 1×10^6 cells/ml (Figure 2). So, according to our result 1×10^5 cells/ml is optimum for growth and sub-sequent propagation of BM-MSCs. Further, validating the data by one way analysis we could interpret that there exists significance differences ($p \leq 0.05$) between the formation of number of colonies due to different seeding density (Table 1).

Table 1: Differences between number of colonies generated due to cell seeded at different densities

Source of variation	SS	df	MS	F	p	F crit
Between groups	3.285E+12	2	1.642E+12	15.92	1.128E-06	3.09
Within groups	9.59E+12	93	1.031E+11			
Total	1.287E+13	95				

4.2. Evaluation of optimum temperature

Different proliferation rate were recorded for cells incubated at 21˚C, 25˚C, 29˚C, 33˚C,37˚C and 41˚C.

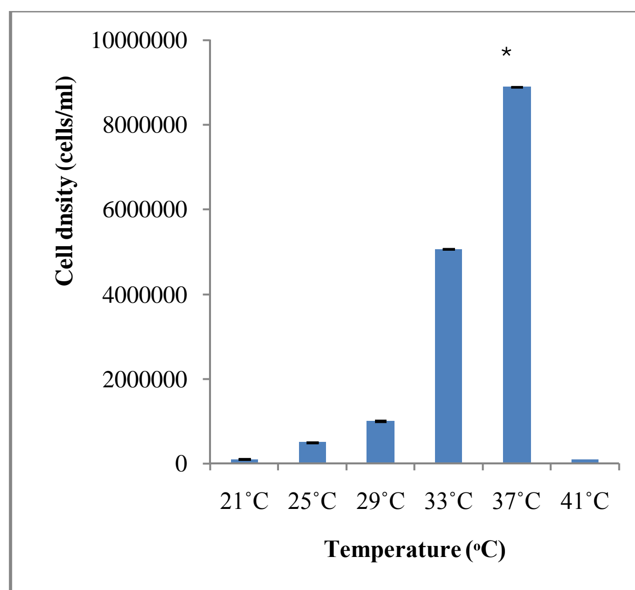

Figure 3 Effect of temperature on MSCs proliferation. *above the column represents significantly different values ($p < 0.05$), expressed as mean ± SEM (n = 16).

It is most important to note that cell proliferation increases as the temperature increases from 21°C to 37°C. However, highest proliferation (8900991.25 ± 4.76) obtained at 37°C in comparison to (5065207.5 ± 1.84) at 33°C, (1001989 ± 2.16) at 29°C, (500096.75 ±1.49) at 25°C, (100054 ± 2.97) at 21°C and (90003 ± 1.47) at 41°C. The data clearly indicate that higher temperature 41°C has detrimental effect on BM- MSCs proliferation.

4.3. Evaluation of optimum FBS concentration

It is evident that from the present study that the cell proliferation was significantly higher ($p \leq 0.05$) in medium containing 10% FBS concentration (25670898 ± 2.94) when

compared with other concentration (2098781± 1.95) at 7%, (1789903.5± 1.32) at 5%, (1566455.5 ± 3.88) at 3%, (809923.5±4.03) and (50026 ± 3.48) at 15 % after cultivating 15 days interval (Figure 4). It was observed that the cell proliferation increases as concentration of FBS increased from 1% to 10%. Further, when concentration of FBS exceeds 10% cell differentiation occurred.

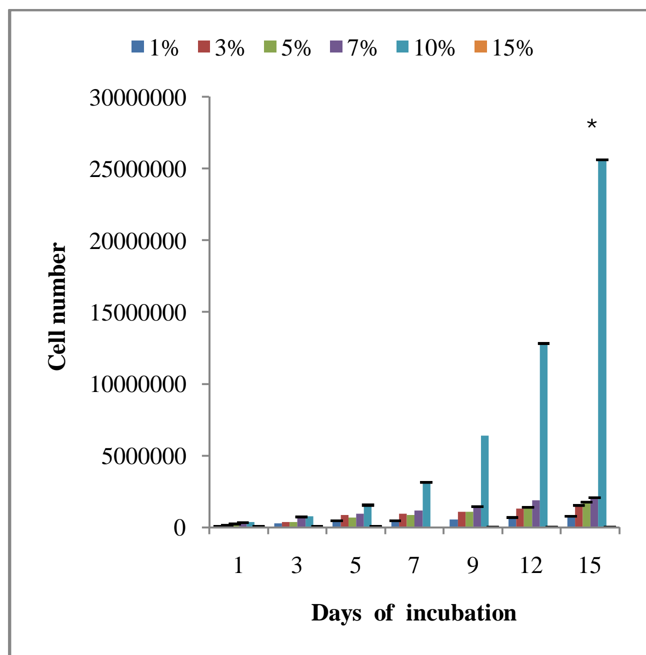

Figure 4: Effect of fetal bovine serum (FBS) on MSCs growth and proliferation. Superscript * above the column represent significantly different value (p < 0.05), values expressed as mean ± SEM (n = 28).

Further, validation of data by one-way Anova clearly demonstrates that there exists significance difference ($p \leq 0.05$) between cell proliferation due to different concentration of FBS as predicted from p value=0.00 (Table 2)

Table 2: Differences between cell proliferation due to different concentration of FBS

Sources of variation	Sum of Squares	df	Mean square	F	Sig.
Between concentrations	1018956263431141.00	5	203791252686228.34	16.05	0.00
Within concentrations	2056441412782139.00	162	12694082794951.47		
Total	3075397676213281.00	167			

Significance difference ($p \leq 0.05$) also exists between the cell proliferation cultured for different days at various FBS concentration.

Table 3. Differences between cell proliferation cultivated for different days at various FBS concentration

Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Between days	472343037976686.800	6	78723839662781.140	4.869	.000
Within days	2603054638236594.000	161	16168041231283.195		
Total	3075397676213281.000	167			

4.4. Evaluation of optimum concentration of growth factor (GMCSF)

GMCSF has significant effect ($p \leq 0.05$) on MSC-like colony formation. It is perceptible to note that significantly higher number of colony formed (180 ± 1.47) at concentration 10ng/ml of GMCSF than other used concentration (96 ± 2.48) at 5ng/ml, (117± 3.93) at 15ng/ml and (101±1.58) (Figure 4).

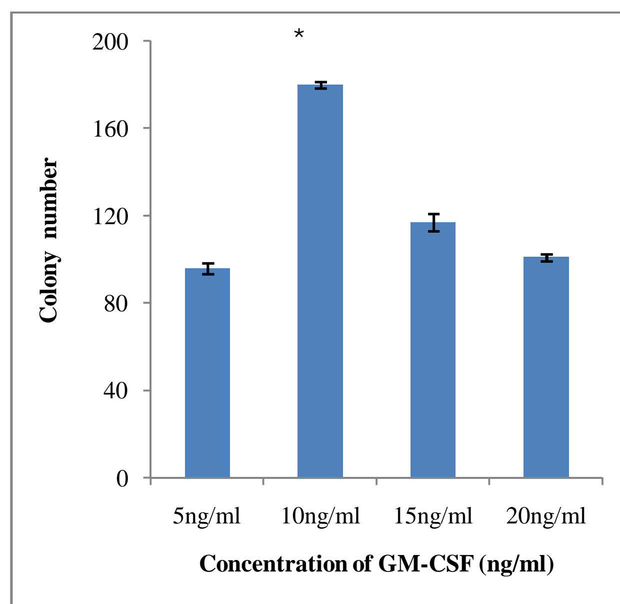

Figure 4 "Effect of GMCSF on MSCs growth. Superscript * above the column represent significantly different value (p < 0.05), values expressed as mean ± SEM (n = 16).

4.5. Growth characteristics of MSCs

From the growth curves analysis it was determined that optimization of factors result good proliferation and expansion along with passages. The calibrated growth curves were plotted for BM-MSCs at P2, P6 and P10 level. All the cells from respective passages exhibited an initial lag phase at day 2, Thereafter, these entered into exponential growth after day 3. It was interesting to notice that the plateau phase was not achieved by cells cultured from P 2 during the time interval 0-

7 days. However, BM-MSCs cultured from P6 and P10 showed declined growth rate after day 6 and entered in the plateau phase (Figure 5). The estimated average PDT for P 2, P6 and P10 was 30.08 hrs, 34.53 hrs and 39.32 hrs respectively. It was observed that PDT of BM-MSCs increases as the passage number increases.

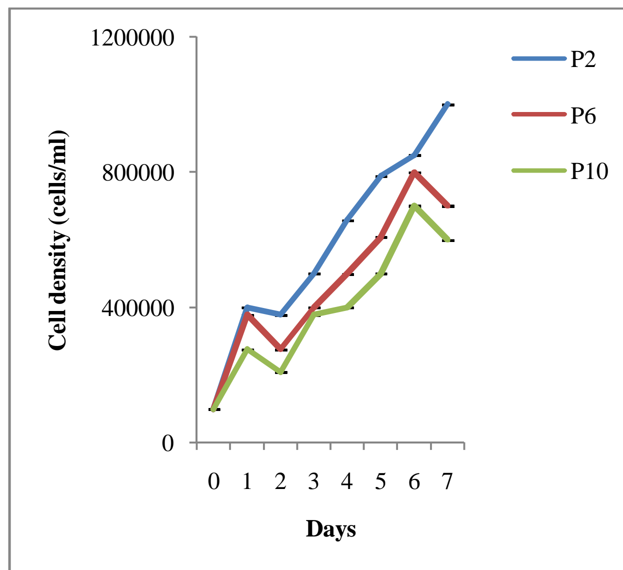

Figure 5: The growth curve plotted for the BM-MSCs from P2, P6 and P10

5. DISCUSSION

MSCs hold great therapeutic promises to treat numerous human diseases and widespread clinical implementation. The presence of considerably lower percentage of MSCs in adult BM is major limitation of using in vast numbers of regenerative and immunotherapeutic approaches {1]. So, *in vitro* expansion of MSCs is highly required to get adequate number before administration to patients. An optimized *in vitro* environment is further needed that facilitates MSCs growth and proliferation maintaining their stemness. The effect of all physical, chemical and biological factors those determine fate and potency of MSCs have not been clearly understood [3]. *In vitro* expansion and growth of MSCs is mediated by several factors and mediators [4]. Cell seeding density is a critical determinant for *in vitro* expansion and exhibit subsequent cellular activities [5]. In this study when isolated MSCs were seeded at the rate 1×10^5 cells/ml in culture generate efficient number of MSC-like colonies. It is further demonstrated that when cell seeded @ 1×10^4 cells/ml lower than 1×10^5 cells/ml insignificant number of colonies results. This may be due to the fact that as the cell number decreases both intracellular and extracellular communication also decreases and the cells fail to aggregate efficiently. In addition when cell seeded at density 1×10^6 cells/ml greater than 1×10^5 cells/ml formed MSC-like colonies unable to sustain in subsequent propagation.. This may be due to the fact that higher cell concentration results fragile colonies. Previously it was observed that when initial plating densities of 5,000 to 10,000 cells/cm^2 results MSC-enriched adherent population [6]. Manipulation of temperature has differential effect on cell proliferation. Decrease in incubation temperature reduce specific growth rate [7]. Here, the higher proliferation of MSCs was noticed at 37°C (physiological temperature). It is well established that proliferation, self-renewal activity and differentiation of *in vitro* cultured stem cells can be improved by mimicking the *in vivo* environment [8]. In this study, it was observed that higher temperature (41°C) has detrimental effect on cells as it inhibits cell proliferation. This may be due to fact that rise in temperature causes sensitive proteins to denature and ceases cell proliferation, whereas at lower temperature slow down catalysis and polypeptide initiation essential for cell proliferation. Our result are in consistent with earlier publish report [9].. FBS is used as chief nutritional supplement by most of the cultured cells [10, 11]. Here, BM-MSCs were optimized with 10% FBS and observed to be more suitable for long-term maintenance. GM-CSF is an autocrine/paracrine cytokine that stimulates colony formation, growth and differentiation [12]. This study revealed that addition of GM-CSF enhances MSC-like colony formation. Similar observation was also noticed by isolated marrow precursor cells to form colonies in semisolid media and to generate colony-forming cells (CFC) in liquid culture on marrow cells on liquid culture [13]. The growth curves of BM-MSCs cultured from P10 shown slower with a shorter exponential growth phase than P2 and P6. It was also observed that BM-MSCs of P2 expanded more rapidly with faster PDT and higher clonogenic potential than P6 and P10. This may be due to the fact that as stem cells age, their self renewal ability and proliferative ability decreases [14]. Similar observation was reported in guinea pig [15]. In the present investigation the estimated PDT was found to be much lower than earlier published report [16]. This may be due to the fact that standardization of biological, chemical and physical factors may enhance the rate of proliferation and cause lower PDT.

6. CONCLUSION

This study demonstrates standardization of biological, physical and chemical factors results a simple and efficient protocol for successful isolation and expansion of BM-MSCs that ends up with homogenous cell populations required for *in vitro* experiments and *in vivo* clinical trials. However, further refinement of other factors associated with *in vivo* condition is needed for *in vitro* cultured MSCs mediated therapeutic interventions.

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