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RESEARCH ARTICLE

Effect of Human Platelet Lysate in Differentiation of Wharton's Jelly Derived Mesenchymal Stem Cells

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Abstract: ***Background:*** Mesenchymal stem cells (MSCs) are highly preferred in clinical therapy for repair and regeneration of diseased tissues for their multipotent properties. Conventionally, MSCs have been cultured in media supplemented with animal derived serum, however, it is ideal to expand MSCs in media containing supplements of human origin for clinical therapy. Currently, a number of human derived products are being studied as an alternative to animal sources. Amongst these, platelet lysate (PL) has gained interest in the culture of MSCs without affecting their phenotypic property.

Objective: In this study, we used various concentration of PL (2.5, 5, 7.5 & 10%) in the growth medium of MSCs to identify the least concentration of PL that could be an effective alternative to animal products.

Methods: MSCs were isolated from Wharton's Jelly by using explant method and expanded in various concentration of PL supplemented medium against the standard FBS containing medium. WJ-MSCs were characterised as per the minimal criteria proposed by International Society for Cell therapy (ISCT), Proliferation study by BrdU assay, gene expression study by qRT-PCR, sterility test for bacteria, Mycoplasma by PCR and endotoxin detection by LAL assay.

Results: Whartons jelly derived MSCs (WJ-MSCs) cultured using standard medium supplemented with various concentration of PL exhibited enhanced proliferation and differentiation potential, unaltered immunophenotypic property and genetic stability when compared with the commercial medium containing 10% FBS.

Conclusion: The least concentration of PL for an ideal expansion of MSCs was found to be 2.5% and was comparable to FBS.

ARTICLE HISTORY

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1. INTRODUCTION

Mesenchymal stem cells (MSCs) due to their pleiotropic potential such as self-renewal, plasticity, ability to differentiate into different cell types of mesodermal origin, immunosuppressive and anti-inflammatory properties have found its way in clinical translation [1, 2]. One translational barrier that limits its clinical use is the incorporation of animal derived supplements specifically fetal bovine serum (FBS) in the culture media for ex-vivo expansion of MSCs [3]. The use of FBS and other animal derivatives have been discouraged by WHO and other regulatory authorities due to several reasons such as xenogenic immune reactions in the host, risk of transmitting prions and zoonoses as well as animal welfare concerns [4-6]. Kievits *et al*. (1988) have reported that side effects such as strong immunoreactions to β2 microglobulin, which originate from FBS supplemented MSC culture medium was observed in animal models following MSC infusion [7]. Hence, efforts to identify alternative growth supplements have come into focus. In this context, human alternatives are much preferred as they can create a culture environment that more accurately resembles the human environment [8]. Moreover, the use of autologous products obviates the need for testing for infectious and other disease causing agents.

Till date, the various human derived supplements studied are autologous and allogeneic human serum, human plasma and platelet derivatives, such as platelet lysate (PL) and release factors [9-12]. Bieback *et al*. (2009) compared the expansion and proliferation of bone marrow derived MSCs (BM-MSC) in different medium enriched with human derived supplements such as human platelet lysate (hPL), thrombin activated platelet rich plasma (tPRP) and human

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serum against FBS [13]. They have reported that all human derived supplements support the isolation and expansion of BM-MSC comparable to FBS with PL being the best.

Platelet lysate (PL) is a rich source of growth factors and cytokines [14, 15]. PL-expanded MSCs have been used in clinical trials for several therapeutic applications with no severe side effects. Such MSCs expanded in PL have been shown to also retain their immunosuppressive properties. Most researchers have used 5% PL for the expansion of MSCs. Though use of PL for the culture of MSCs has been initiated nearly a decade ago [16], there is no clear data available regarding the ideal concentration for the efficient proliferation and differentiation of MSCs in PL medium.

In our previous work, we have compared the MSCs expanded in 10% FBS and 10% PL medium (data not shown) and found that 10% PL was much superior. In order to get the large scale expansion of MSCs for clinical translation, the requirement of culture medium will be more. Hence, the purpose of our study was to find the least appropriate concentration of PL medium in which MSCs can proliferate rapidly while maintaining their immunophenotype and differentiation ability. In order to standardise the ideal concentration for the growth of MSCs we cultured MSCs in DMEM supplemented with various percentages of PL (2.5, 5, 7.5 and 10%) and compared with the DMEM containing 10% FBS.

2. MATERIALS AND METHODS

2.1. Sample Collection

A segment of the umbilical cord approximately 8-10 cm in length was aseptically collected at the time of delivery in sterile PBS, from mothers who had given informed consent prior to the planned elective Caesarean Section at RSRM Hospital, Chennai, Tamilnadu, India. The sample was transported to the Stem cell research lab immediately (less than half an hour) and processed immediately.

2.2. Preparation of DMEM with Pooled Platelet Lysate (PL)

Methods described by Hemeda *et al*. (2014) were used to prepare hPL [11]. The blood bank of Stanley hospital provided the platelet concentrates (PC) 5 -7 days old from 5 donors after informed consent and meeting the regulatory requirements for sterility and infectious disease screening applicable for transfusion products. PC was subjected to freeze thaw procedure for 3 consecutive days, *i.e*. freezing at -80° C for 24 h followed by thawing at 37° C for 1 h. All 5 PC were then pooled into a single sterile container, mixed well and centrifuged at 4000g for 15 mins at 4 ^{0}C. The supernatant was filtered through 0.2µm filter, aliquoted and stored at -80° C. The aliquot was then used to prepare 2.5, 5, 7.5 and 10% concentrations in DMEM into which 4 IU/ml of heparin was added (to prevent gelatinisation).

2.3. Preparation of DMEM with Platelet Lysate from a Single Donor

One unit of platelet concentrate (5 -7 days old) from a single donor that meets all regulatory requirements for sterility and infectious disease screening for transfusion products were collected from Stanley hospital blood bank after informed consent. The PL of this single donor was also prepared using the same method as for pooled PL.

2.4. Mesenchymal Stem Cell (MSC) Isolation

DMEM with various percentages of platelet lysate (2.5% - , 5% - , 7.5%- and 10% PL) in T75 flasks as well as DMEM supplemented with 10 % FBS to serve as a standard control were used to culture MSCs following the standardised protocol as described [17].

2.5. Immunophenotypic Analysis by Flow Cytometry

Each passage of MSCs (passages 1- 5) was characterized by determining the cell surface antigen expression using fluorescent conjugated antibody panel from BD Biosciences: anti-human CD90-FITC, CD73-APC, CD105-PerCp-Cy5.5 (mesenchymal lineage) and CD14-FITC, CD34-PE and CD45-APC-CY7 (hematopoietic lineage). Flowcytometry was performed on BD Facs Aria^{TM} II Flow cytomter and results analysed using DIVA software Version v 6.1.2.

3. GROWTH KINETICS

3.1. Growth Curve

MSCs were seeded in 6-well plates at the density of 1×10^4 cells/well and cultured using various concentrations of PL (2.5%, 5%, 7.5% and 10%) following the method mentioned above. Duplicates of MSCs were harvested after trypsinisation every second day until day 12. Viability was tested by trypan blue dye exclusion. The counts obtained were plotted to generate a growth curve. The experiments were performed in triplicates.

3.2. BrdU Cell Proliferation Assay by Flow Cytometry

Cell proliferation was evaluated by using the thymidine analog BrdU (5-bromo-2-deoxyuridine from Pharmingen Flow kit, BD Biosciences, San Diego USA) following manufacturer's instructions. Briefly, when the cells reached 60-70% confluency, cells were labelled with 10µM concentration of BrdU and incubated for 24 h. The cells were fixed and permeabilised using BD cytofix/cytoperm buffer in kit, followed by treatment with DNase to unwind the DNA and help the anti-BrdU antibody to access the incorporated BrdU. For staining total DNA in viable cells, 7-amino-actinomycin D (7-AAD) was used. The 7-AAD population was gated and BrdU positive cells were analyzed by flow cytometry as an index of proliferating cells. BrdU unlabeled cells served as negative control. The experiments were performed twice in triplicates.

3.3. Population Doubling Time (PDT)

For this, MSCs were seeded at a density of 5000 cells/cm^2 in T-25 flask and cultured as per the above mentioned condition. When the cells reached confluency (100%), the flasks were trypsinized and viable counts recorded. Doubling time (Td) was determined using initial seeding density (N_0), number of hours to reach confluency (T), and cell yield at confluency (N_t), using the standard Patterson Formula [T_d = Tlg2/lg (N_t/N_0)] [18]. The doubling

time of each group (4 concentrations of PL and 10% FBS) was obtained.

3.4. Colony Forming Unit (CFU) Assay

MSCs (passage 2) on reaching 70-80% confluency were trypsinised and diluted using complete medium to seed 1000 Cells/100 mm petri dishes and incubated at 37°C at 5% CO_2 for 12 days. On day 12, the culture plate was washed with PBS twice and stained for 10 mins with 0.5% crystal violet prepared in methanol. The stained colonies were washed with PBS and visible colonies were counted [19].

3.5. Trilineage Differentiation Assays

3.5.1. Osteogenic Differentiation

MSCs (10000 cells/well; passage 2) cultured using various concentrations of PL and 10% FBS were differentiated to osteogenic lineage. 10^{-8} M dexamethasone (Sigma, D4902), 10 mM β glycerophosphate (Sigma, G9422), and 50 µg/ml ascorbic acid was added to the DMEM supplemented with various concentrations of PL/10% FBS to render it osteogenic. Media was changed for every 48 h. At day 12, the mineralization/calcium deposits in cultures were detected by staining with Alizarin Red S (Sigma) and compared with undifferentiated controls. The experiments were performed twice in triplicates.

3.5.2. Adipogenic Differentiation

MSCs (10000 cells/well; passage 2) were cultured using various concentrations of PL and 10% FBS were differentiated to adipogenic lineage. 10 mM 3- isobutyl-1-methylxanthine (Sigma, 17018), 0.1 mM indomethacin (Sigma, 17378), 10 µg/ml insulin (Sigma, I6634), 10^{-6} dexamethasone was added to the DMEM supplemented with various concentrations of PL/10% FBS to render it adipogenic. Media was changed for every 48 h. At day 21, oil droplets of adipocytes differentiated from MSCs were confirmed by Oil red O staining for all the groups and compared with respective controls. The experiments were performed twice in triplicates.

3.5.3. Chondrogenic Differentiation

MSCs (Passage 2) cultured using various concentrations of PL and 10% FBS were differentiated to chondrogenic lineage by micro mass culture method. 5 µl of 1.6 x 10^7 cells /ml were seeded at the centre of 6 well plates and incubated at 37° C in 5 % CO_2 for 2 h to create micromass. After 2 h, commercial chondrogenic medium (STEMPRO, A1007101) was added and incubated. Control cells were micromass of MSCs cultured only with DMEM supplemented with 10% FBS. Media was changed for every 3 days. At day 21, Toluidine blue staining was used to check for chondrogenic tissue and compared with controls. The experiments were performed twice in triplicates.

3.5.4. Gene Expression Studies to Assess the Levels of Adipogenic, Osteogenic and Chondrogenic Differentiation Markers Using qRT-PCR

For gene expression studies, total RNA was extracted from the MSCs (passage 2) differentiated into adipogenic, osteogenic and chondrogenic lineages for 21 days in the various percentages of PL supplemented medium by Trizol method. cDNA conversion was performed using BIORAD cDNA synthesis kit. Expression studies for the following genes PPARγ, Adiponectin, RunX2 for adipogenic differentiation, Osteocalcin, Col 1A1 for osteogenic differentiation and Sox 9 for chondrogenic differentiation was assessed by qRT-PCR using β-actin as internal control. Data were expressed as relative expression.

3.5.5. Gene Expression Studies to Assess the Stemness of MSCs Using RTPCR

For gene expression studies, the MSCs of passage 2 were cultured and expanded at 2.5, 5, 7.5, 10% PL and 10% FBS. Total RNA was extracted using Trizol method followed by cDNA conversion performed using BIORAD cDNA synthesis kit. cDNA was assessed for the expression of the following genes Oct4, Nanog and Sox 2 using conventional PCR.

3.5.6. G-banded Karyotyping

Colcemid (10 mg/ml) was added to each flask at a final dilution of 0.1µg/ml upon 80% confluency on culture and further incubated at 37°C for 30 mins. Changes in cell morphology were observed by microscopy. After trypsinisation cells were centrifuged at 400×*g* for 10mins. Hypotonic treatment was done as follows: 10 ml of 0.075 M KCl was added slowly, incubated at 37°C for 30 mins and fixed with methanol: acetic acid (3:1) solution. The cell suspension was dropped on to precleaned cooled slide from a height for uniform blast. The slides were baked at 80°C for 1 h and then placed at 37°C for at least16 h, immersed in trypsin solution (0.002g/mL) for 5 seconds, washed in sorenson's buffer and finally quickly rinsed in distilled water. Staining was performed using Giemsa dye (1: 20). Trypsin and Giemsa (GTG) bands were studied by microscopy (100X). At least 20 metaphases were analyzed using Applied Spectral Imaging (ASI) software to obtain the karyogram.

3.5.7. Sterility & Endotoxin Assays

Bacterial culture methods were used for sterility testing of all MSCs cultured at various concentrations of PL and 10% FBS. Mycoplasma screening was performed by two step Nested PCR based on 16S and 23S rRNA sequence (TaKaRa, Cat. No.6601). Endotoxin assay was done using Limulus amoebocyte lysate (LAL) assay (Lonza QCL1000).

The cell culture supernatants of MSCs expanded in all groups were free of bacteria and mycoplasma. Endotoxins in the various groups were found to be much less than the permissible range. (< 0.05 EU/ml)

3.5.8. Statistical Analysis

All comparisons were performed by one-way ANOVA and Tukey's test and a p value of less than 0.05 was considered significant.

4. RESULTS

4.1. Morphological Analysis

Spindle shaped fibroblast like morphology was observed in MSCs expanded in all groups with some morphological differences observed in the following groups. MSCs

expanded in PL medium (all concentrations) showed densely packed, smaller but broader spindle cells while MSCs expanded in FBS medium showed loosely attached narrow spindle cells (Fig. **1**).

4.2. Immunophenotypic Profiles

No significant difference in MSC surface markers was observed by flow cytometry analysis between the five different types of medium. In all groups, MSCs were positive for CD73, CD90 and CD105 and negative for hematopoietic markers CD14, CD34 and CD45 as per the ISCT criteria (Figs. **2**, **3**).

4.3. Growth Kinetics and Population Doubling Time (PDT)

Growth kinetics and PDT of MSCs in various concentrations of PL and 10% FBS medium is illustrated in Figs. (**4**, **5**). Briefly, the lag phase for 2.5% PL and 10% FBS was shorter from day 1-4 and longer log/exponential phase from day 4-10 and the doubling time was found to be 31 h for both. In 5% PL, lag phase was observed from day 2-6 followed by log phase from day 6-10 until the plateau was reached and the doubling time was 25 h. In 7.5% PL, the lag phase was observed between day 2-6 and the shorter log phase observed between day 6-8 and the doubling time was found to be 22 h. Similarly, in 10% PL, the lag phase was observed between day 2-4 and log phase was observed between day 4-8 and the doubling time was found to be 23 h.

4.4. BrdU Cell Proliferation by Flow Cytometry

The highly proliferative cells indicated at S phase were maximum in 7.5% PL medium (95%) whereas 5 % PL and 10% PL showed 84.7% and 81.8% respectively in S phase. 2.5% PL showed 46% proliferation in S phase and 10% FBS had 35.3% proliferation (Fig. **6**).

4.5. Colony Forming Unit (CFU) Assay

The number of CFU in 2.5% (CFU -113) was similar to 10% FBS (CFU 109). Higher CFU counts of 181,195, and 226 were observed with higher concentrations of PL of 5%, 7.5% and 10% respectively (Fig. **7** and Table **1**).

4.6. MSCs in DMEM with PL Derived from Single Donor

MSCs expanded using single donor also showed the similar results as that of pooled PL.

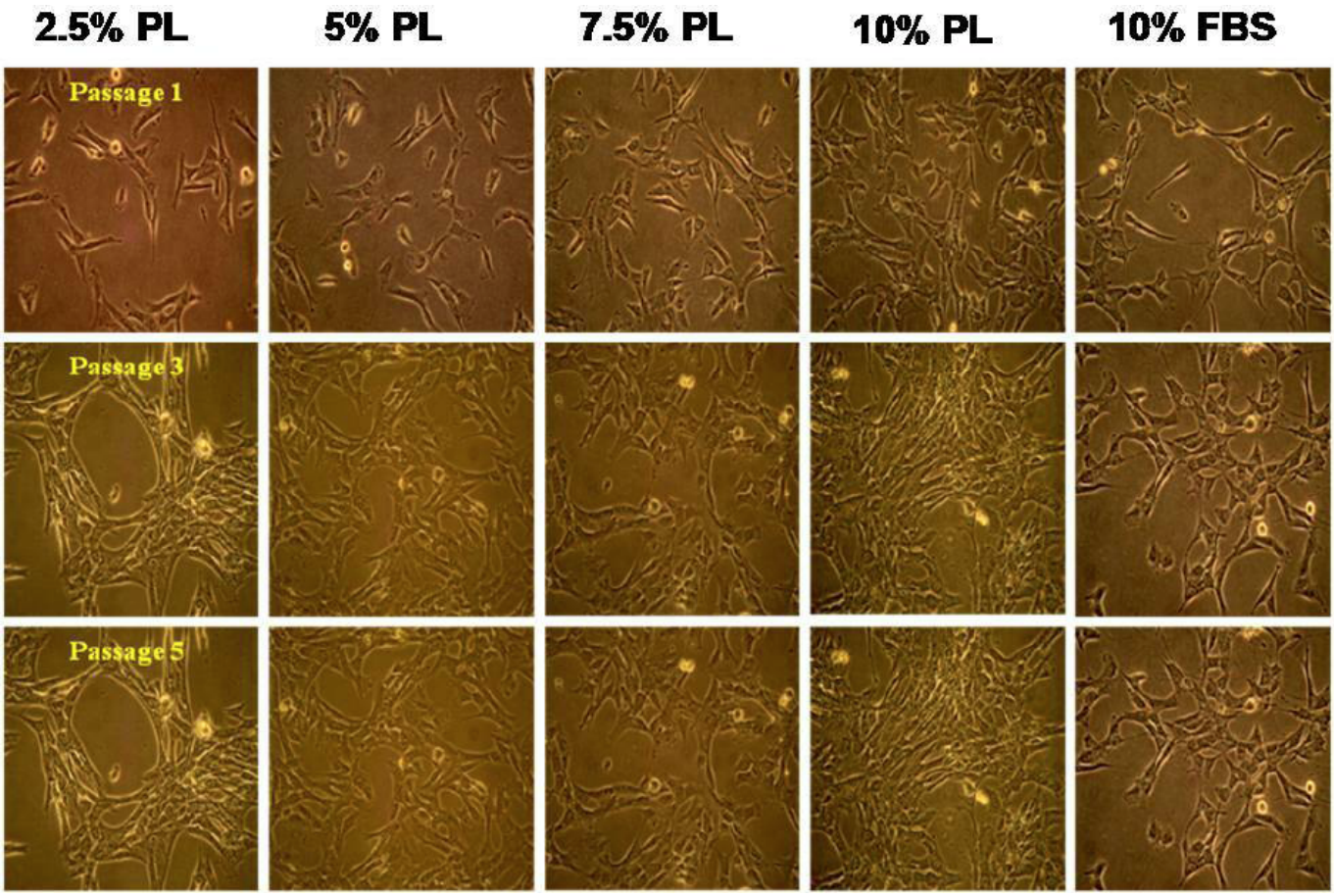

Fig. (1). Morphological analysis of MSCs expanded in various percentages of Platelet lysate (2.5, 5, 7.5, 10%PL) and FBS (10%) supplemented medium.

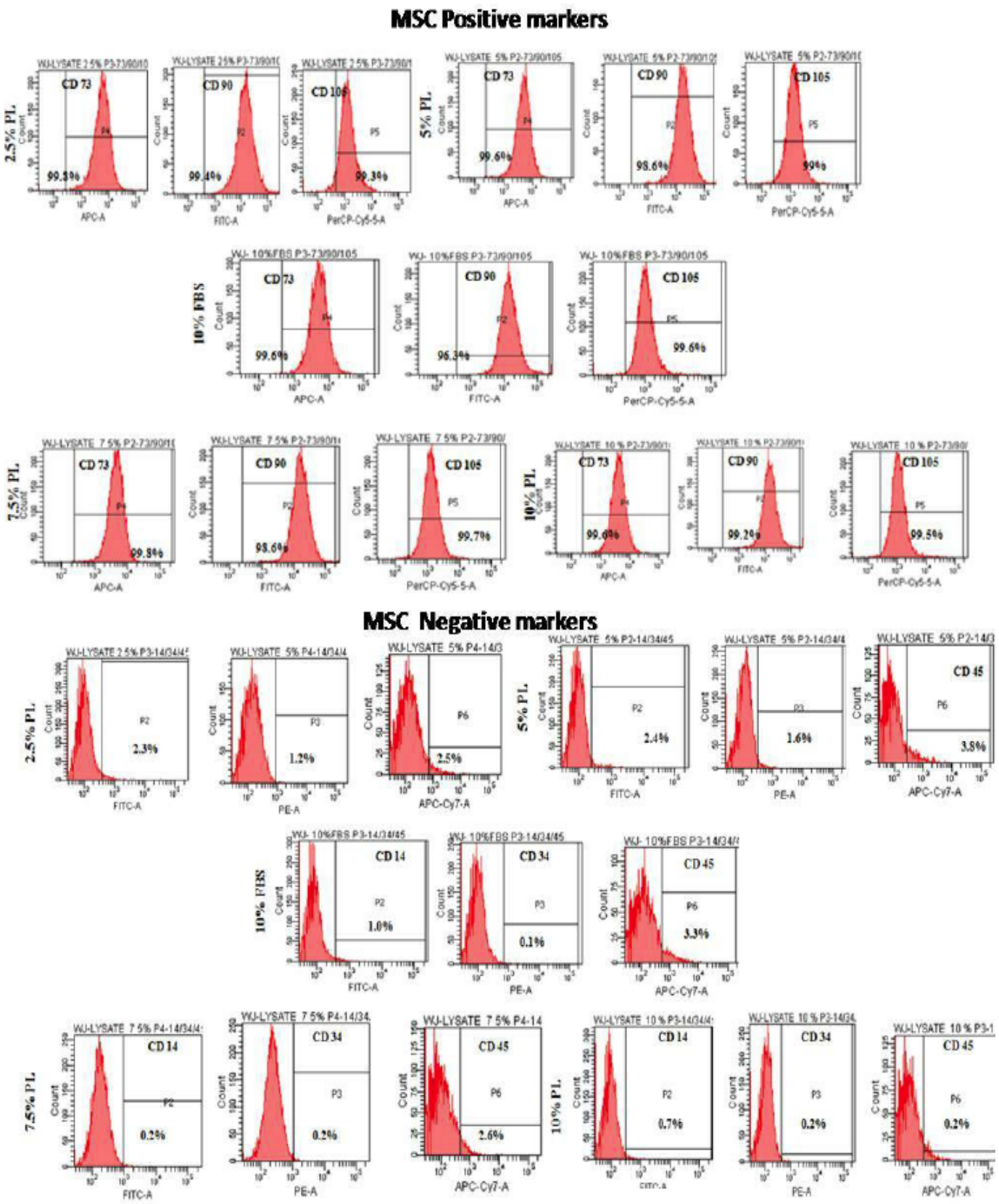

Fig. (2). Immunophenotypic analysis of MSCs by Flow cytometry (**a**) Positive expression of mesenchymal stem cell markers (CD105, CD73, CD90) and (**b**) Negative expression of hematopoietic markers (CD45, CD34 and CD14).

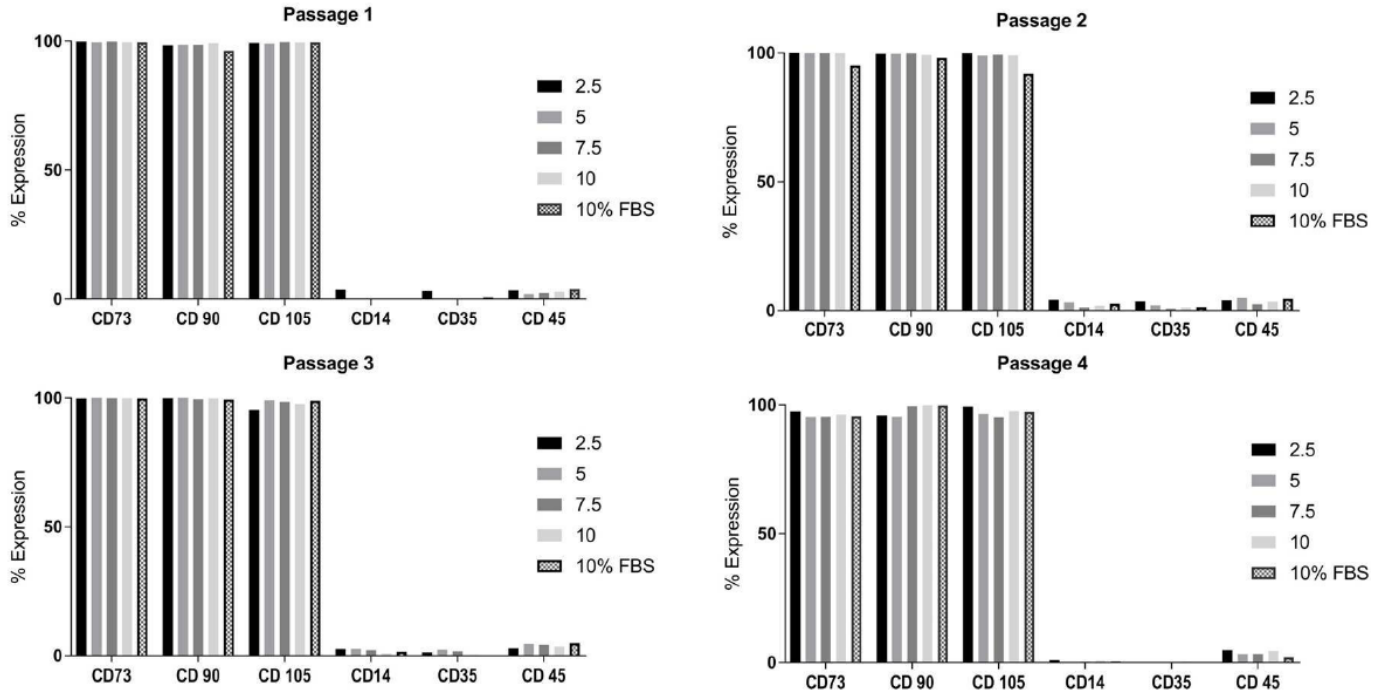

Fig. (3). Graphical representation of surface antigen expression for MSCs by flow cytometry.

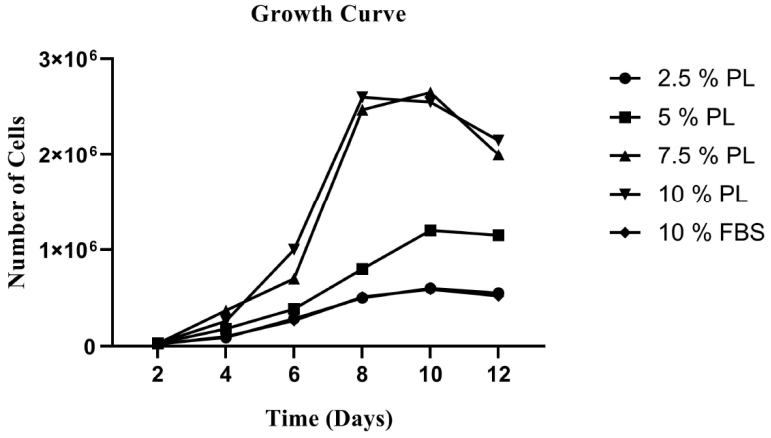

Fig. (4). Growth kinetics of MSCs in various percentages of PL and FBS supplemented medium.

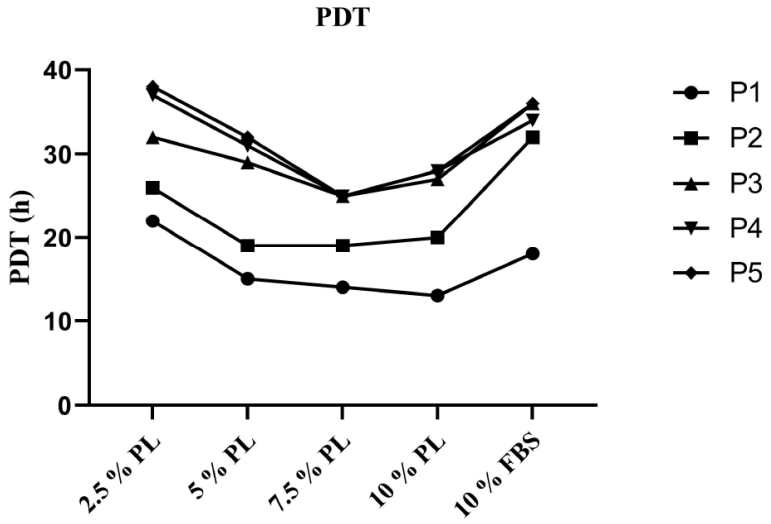

Fig. (5). Population doubling time of MSCs in various percentages of PL and FBS supplemented medium.

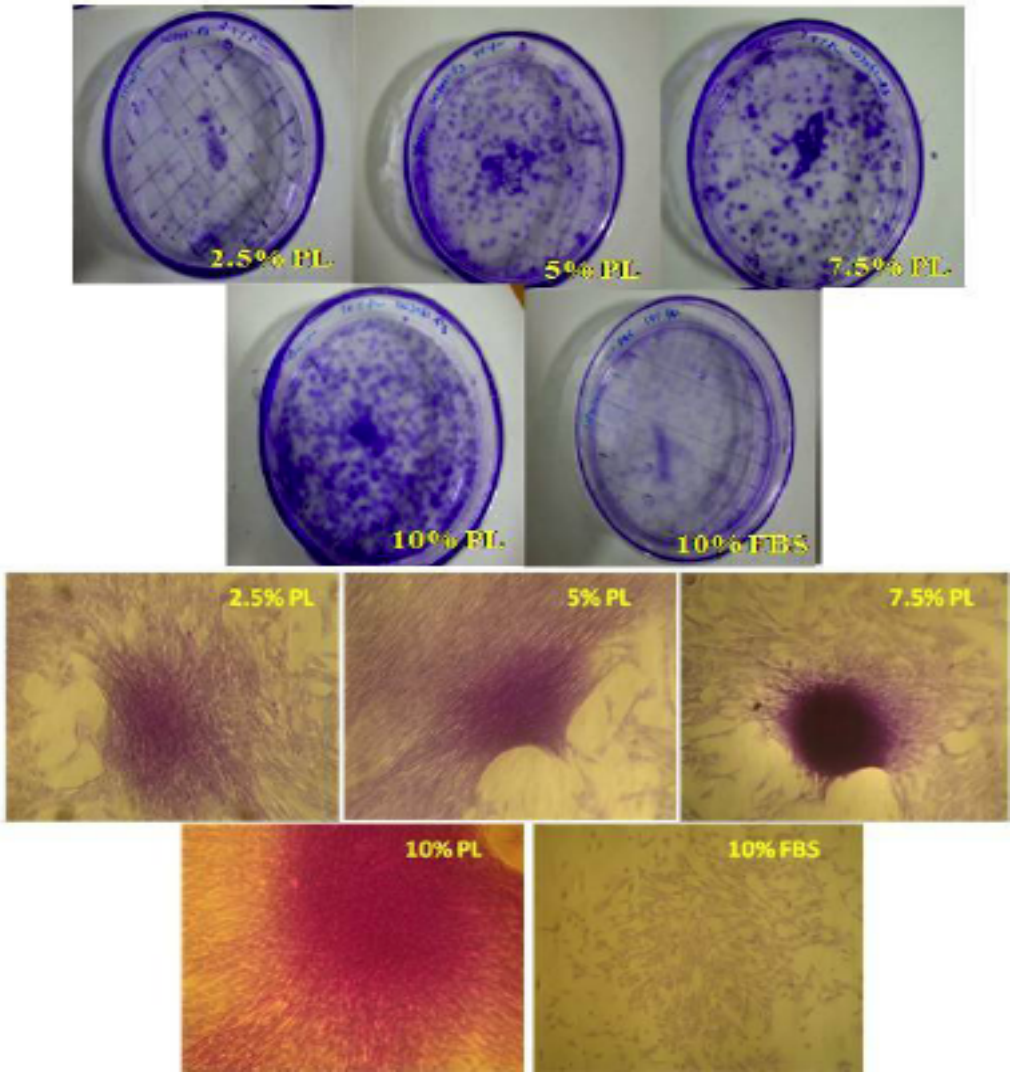

Fig. (6). Proliferative potential of MSCs by BrdU cell proliferation using Flow cytometry.

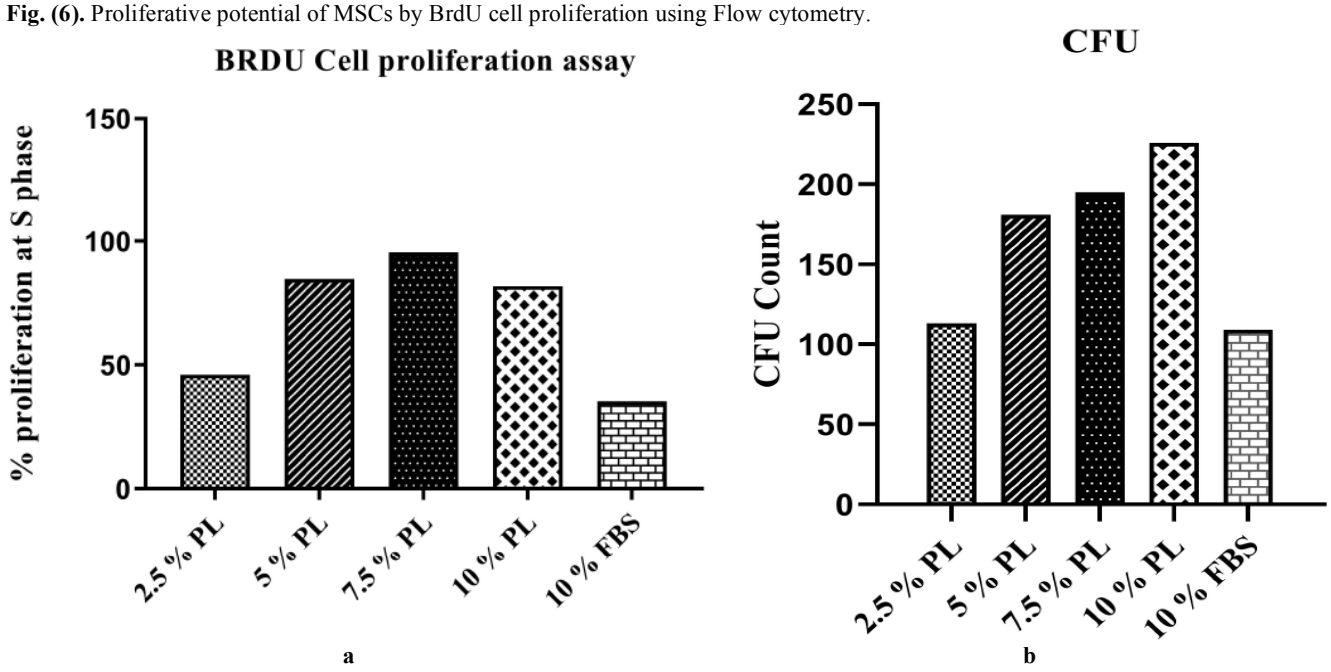

Fig. (7). Colony forming unit assay of MSCs in various percentages of PL and FBS supplemented medium (**a**) CFU staining by crystal violet (**b**) Microscopic images of stained CFU(c) graphical representation.

Table 1. CFU analysis for MSCs in varied composition of DMEM supplemented with PL/FBS.

Composition of PL/FBS in DMEM	CFU Count of MSCs
2.5% PL	113
5% PL	181
7.5%PL	195
10%PL	226
10%FBS	109

5. TRILINEAGE DIFFERENTIATION

5.1. Qualitative Analysis by Specific Staining

MSCs of passage 2 cultured in various concentrations of PL as well as 10% FBS supplemented media were exposed to osteogenic, adipogenic and chondrogenic differentiation conditions as mentioned above. Osteogenic differentiation was observed using alizarin red staining (Fig. **8a**), adipogenic differentiation was observed by Oil red O staining (Fig. **8b**) and chondrogenic differentiation by micromass culture system was observed using toluidine blue staining (Fig. **8c**).

The day of initiation of differentiation are given in Table **2**.

5.2. Quantitative Analysis by Gene Expression Studies Using qRTPCR

The expression of genes involved in the adipogenic (Adiponectin, PPARγ and RunX2), osteogenic (Osteocalcin and Col 1A1) and chondrogenic (Sox 9) differentiation pathways for MSCs differentiated at various concentrations of PL and 10% FBS are as follows.

In the osteogenic differentiated cells, for the Col A1 gene (Fig. **8d**), maximum expression was observed at 2.5% of PL itself. The gene expression decreased with increase in concentration of PL But, on increasing the concentration of PL the gene expression started decreasing indicating a concentration dependant decreasing effect. However, for osteocalcin there was a concentration dependant increase in the gene expression that was initiated at 2.5% PL but was maximum at 10% of PL (Fig. **8e**).

From the results, it was observed that MSCs differentiated at all the concentrations of PL showed increase in adiponectin levels compared to the undifferentiated controls (Fig. **8f**). But the expression was significantly high at 7.5% of PL. In the PPARγ expression studies, 2.5% of PL showed maximum expression compared to 5%, 7.5% and 10% of PL (Fig. **8g**). Likewise, for the RunX2 gene, increase in expression was observed at 5% and 10% of PL whereas 2.5% and 7.5% of PL showed no expression or expression equivalent to that of undifferentiated controls (Fig. **8h**).

For the chondrogenic differentiated cells, Sox 9 gene expression was found to be maximally expressed at 2.5 %and 5% of PL (Fig. **8i**). Whereas at 7.5% and 10% there was an increase in gene expression but was comparatively less compared to 2.5%and 5% of PL.

5.3. Stemness Gene Expression

Expression of the stemness genes Oct4, Sox 2 and Nanog were expressed in all of the concentrations of PL and 10% FBS supplemented media as shown in Fig. (**9**). However, 2.5% of PL showed a maximum upregulation in expression of all the stemness genes. All gene expressions were normalized using β-actin as internal control.

5.4. Karyotype Analysis

MSCs (passages 2 – 5) cultured in medium of all groups were karyotyped. A normal diploid karyotype was observed *i.e*., 46 XY with all groups (Fig. **10**).

5.5. Sterility Test and Endotoxin Assay

The cell culture supernatants of MSCs expanded in all groups were free of bacteria and mycoplasma. Endotoxins in the various groups were found to be much less than the permissible range.

6. DISCUSSION

The present work addresses the interest to identify an optimal human-derived supplement–(Human platelet lysate) as a replacement for animal-derived FBS during the culture and expansion of human adult MSCs. Earlier studies have shown that MSCs derived from different sources and cultured in various concentrations of PRP showed differences in their proliferative capacity. AT-MSCs cultured in 1% PRP, WJ-MSCs in 2.5% and BM-MSCs in 5% PRP were found to be comparable to 10% FBS [20]. The study has also reported that cells grown at higher concentrations of PRP (40% and 50%) attained senescence earlier which was speculated due to the presence of PRP inhibitory factors at high concentration of PRP. Also, PRP is susceptible to changes in temperature during storage creating a possibility for rupture of some of the intact platelets thereby altering the composition of the derived products. Inspite of the emerging use of hPL as an alternative for FBS in the ex vivo expansion of MSCs, key questions such as the lack of a standardised protocol for the preparation of platelet derived supplement and the optimal platelet concentration for MSC growth needs to be addressed to support its clinical use. Hence, it would be valuable to determine the appropriate concentration of platelet lysate (PL) as well as a suitable protocol for the preparation of these humanised substitutes for generation of clinical grade MSCs. Therefore, in our study, Wharton's Jelly (WJ)-derived MSC expansion was performed in medium supplemented with various concentrations (2.5%, 5%, 7.5% and 10%) of human platelet lysate (hPL) prepared from platelet concentrate and was compared with 10% FBS supplemented medium as a standard reference control.

MSCs cultured in hPL supplemented medium were observed to form colonies of densely packed small spindle cells

Figure 8 A. Osteogenic differentiation (Alizarin red staining)

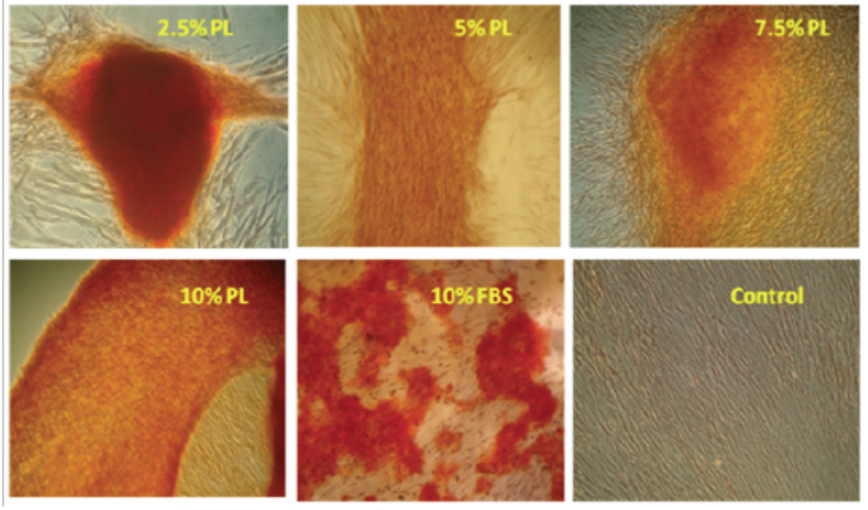

Figure 8 B. Adipogenic differentiation (Oil red staining)

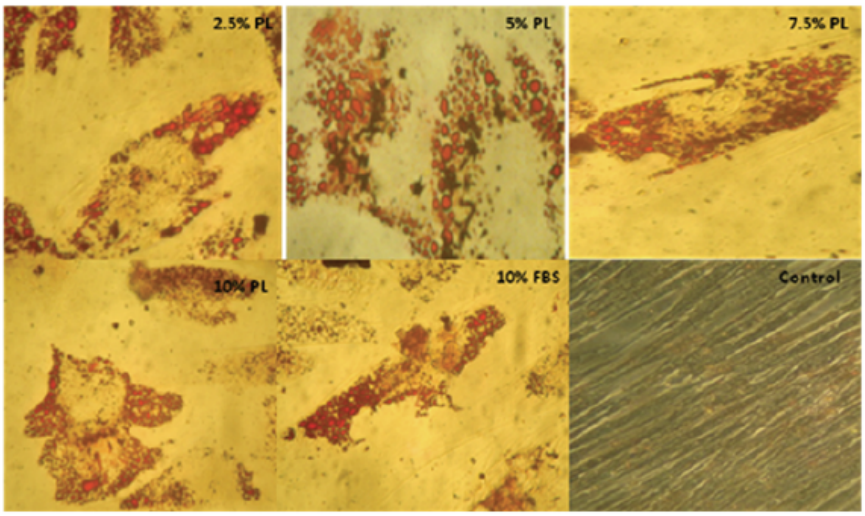

Figure 8 C. Chondrogenic differentiation (Toludine blue staining)

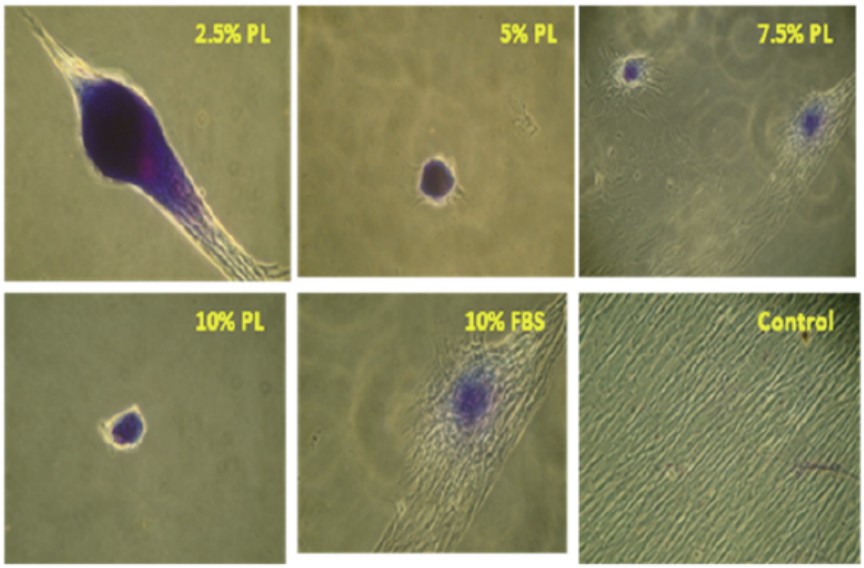

Fig. (8) contd….

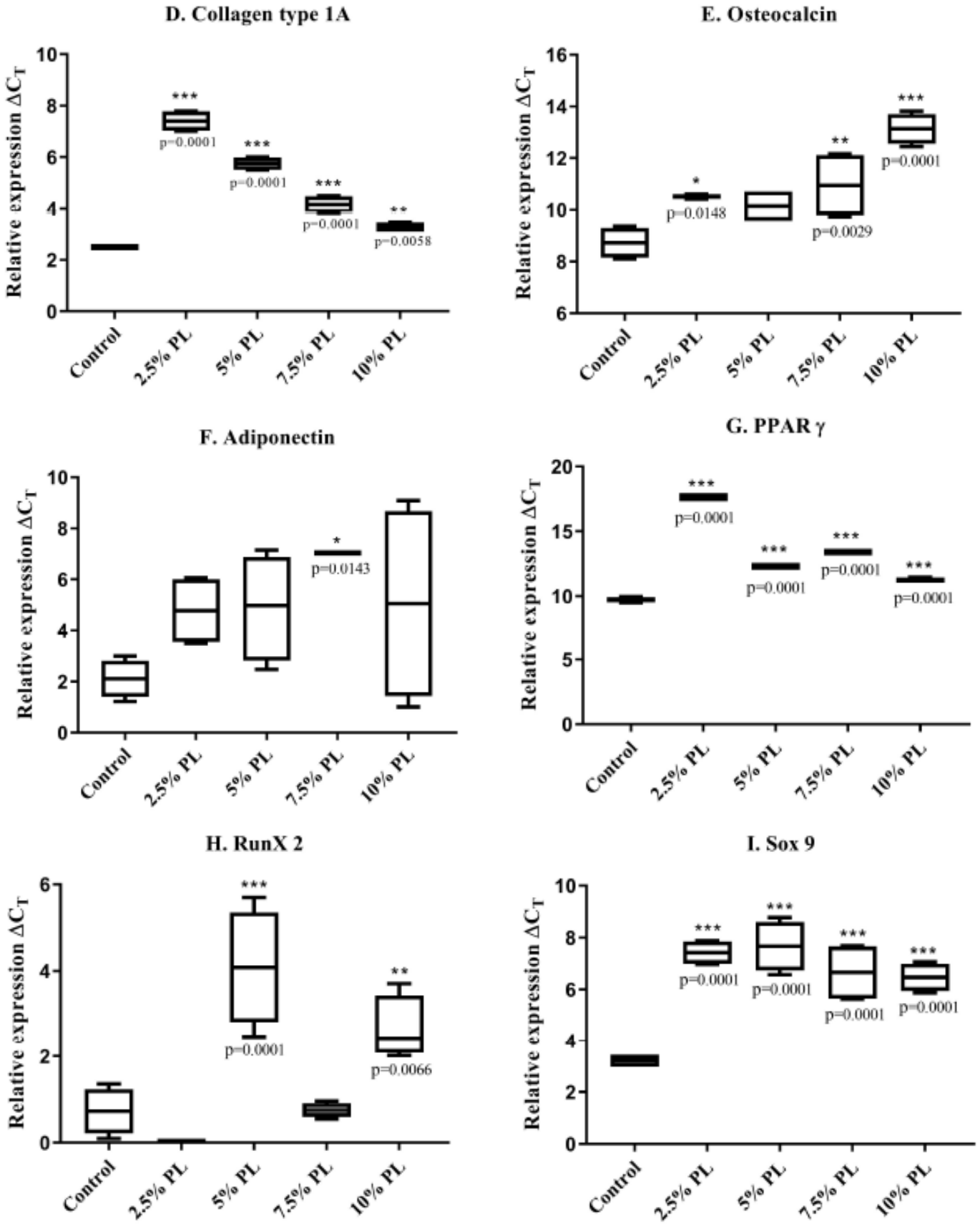

Fig. (8). Differentiation of MSCs to osteogenic, adipogenic and chondrogenic lineages by specific staining (**a**) Alizarin red, (**b**) Oil red O and (**c**) Toludine blue staining. Gene expression studies for osteogenic, adipogenic and chondrogenic differentiated cells (**d**) Col1A, (**e**) Osteocalcin, (**f**) Adiponectin, (**g**) PPARγ, (**h**) RunX2 and (**i**) Sox 9.

Table 2. Days of initiation of Adipogenic and Osteogenic differentiation and number of micromass formed during Chondrogenic differentiation by MSCs cultured and differentiated using PL supplemented medium.

Media Supplementation	Day of Initiation of Differentiation		Micromass Pellet Numbers
	Osteogenic	Adipogenic	Chondrogenic
10% FBS	Day 14	Day 12	5
2.5% PL	Day 12	Day 16	4
5% PL	Day 10	Day 16	2
7.5% PL	Day 7	Day 14	2
10% PL	Day 7	Day 12	3

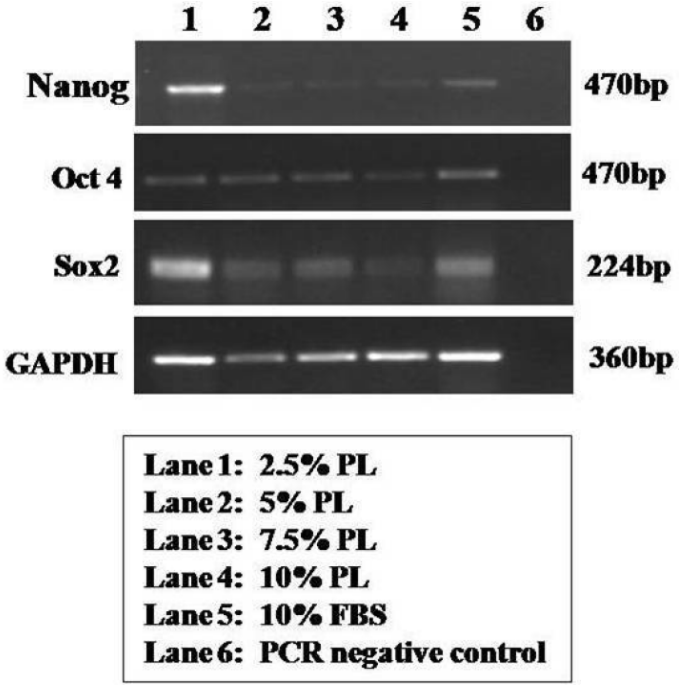

Fig. (9). Gene expression studies for stemness markers like Oct4, Sox 2 and Nanog.

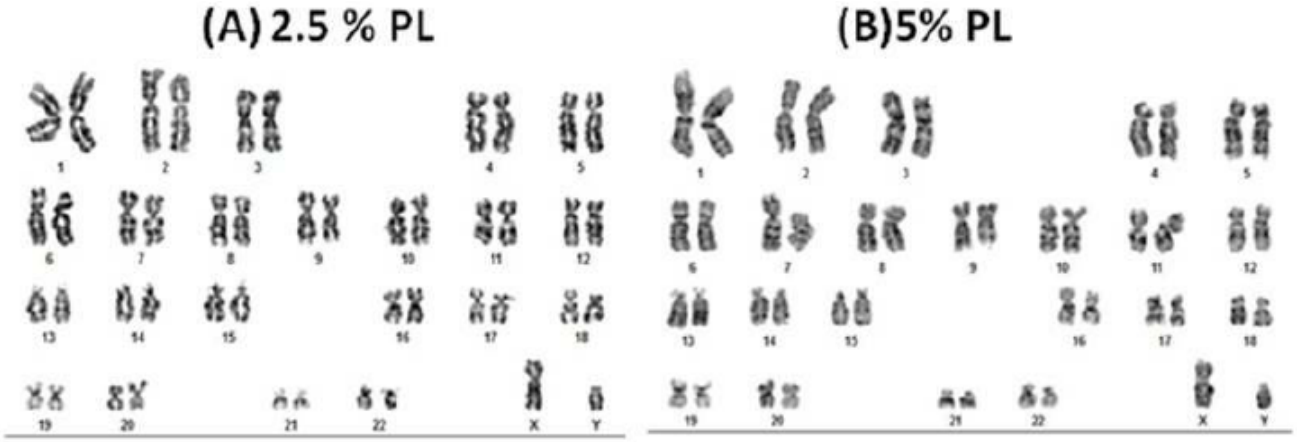

Fig. (10) contd….

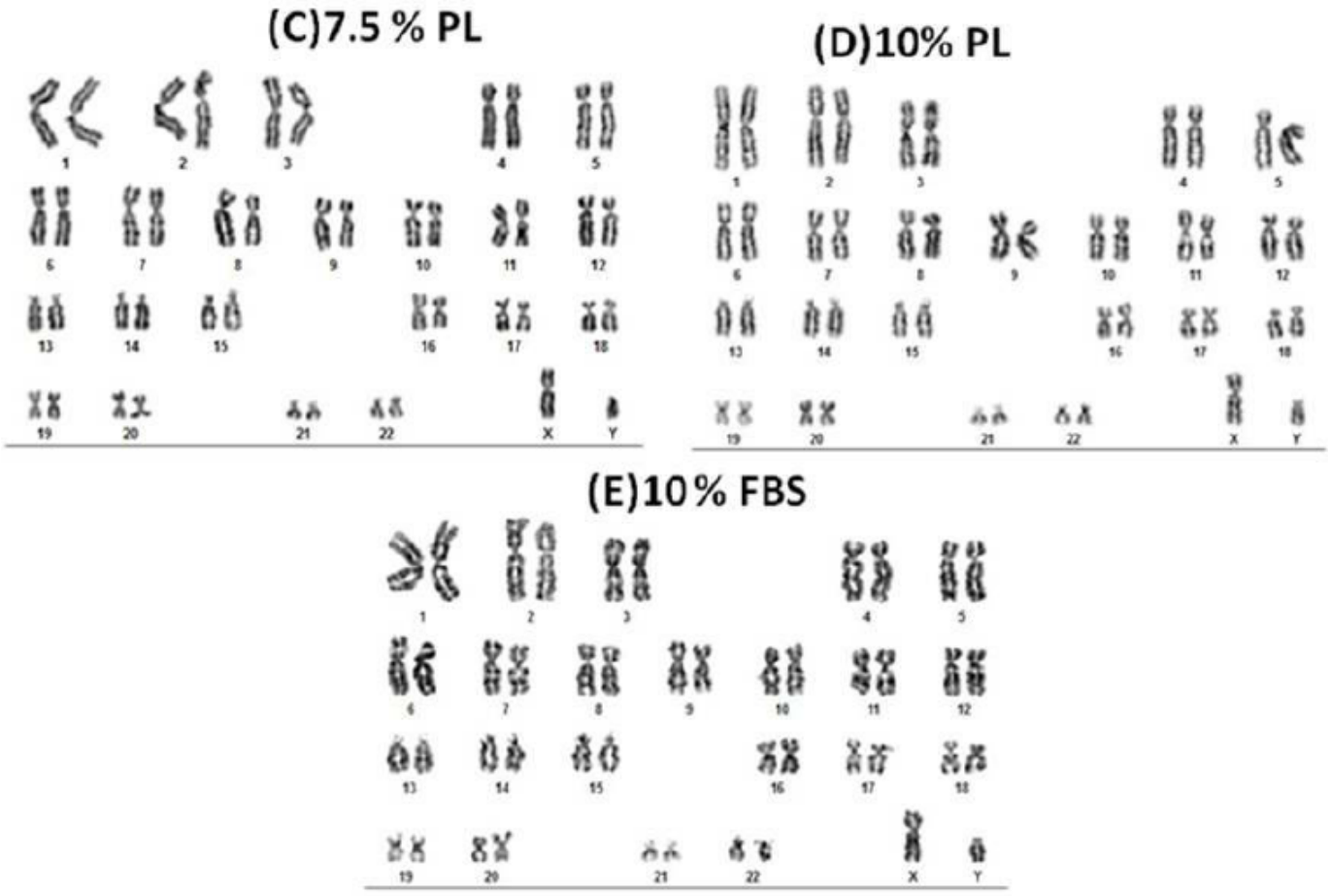

Fig. (10). Karyogram of MSCs in different concentration of PL and FBS supplemented medium.

whereas MSCs cultured in FBS supplemented media appeared to be loosely attached with longer spindle cells. Ben Azouna *et al*. (2012) and Patrick Horn *et al*. (2010) have also reported that the morphology of MSCs was of larger colonies of densely packed small spindle cells compared to colonies of MSCs expanded in FBS containing medium [8, 21]. However, among the cells cultured at various concentrations of hPL supplemented medium, we observed that MSCs expanded in 5%, 7.5% and 10% of hPL supplemented medium were found to rapidly proliferate and attain confluency earlier compared to MSCs expanded in 10% FBS supplemented medium while MSCs expanded in 2.5% hPL supplemented medium were similar to MSCs expanded in 10% FBS supplemented medium.

Cultivation condition and variations in concentrations of media supplements have an impact on the cell doubling time. Lu *et al*.(2006) have reported that the doubling time was shorter for MSCs expanded with humanised substitute supplemented medium than the MSCs expanded in FBS/fetal calf serum (FCS) supplemented medium. Also, the doubling time of Umbilical cord derived MSCs was found to be constant *i.e*. 24 h in all passages (1-10) [4]. In our study, we observed the mean doubling time to be shorter *i.e*., 25,22, and 23 h for 5%, 7.5% and 10% PL respectively whereas for 2.5% PL and 10% FBS the mean doubling time was 31 h for up to passage 5.

Similarly, the proliferation potential was maximum for MSCs expanded in 7.5% PL exhibiting 95.4% of cells in the S phase whereas 5% and 10% PL showed 84.7% and 81.8%. But, 2.5% of PL and 10% FBS showed only 46% and 35.3% respectively. Thus, the proliferation efficiency of even the low concentration (2.5%) of PL was better than 10% FBS while proliferation at concentrations of 5%, 7.5% and 10% PL was further enhanced. The number of CFUs formed in 5%, 7.5% and 10% PL (181,195,226 respectively) was more compared to 2.5% PL and 10% FBS (113 and 109 respectively) which showed almost equivalent colonies. However, the morphology and number of cells in each CFU at all of the concentrations of hPL supplemented medium was found to be dense having small spindles compared to cells in FBS medium. Bieback *et al*. 2009 have also observed densely packed colonies of cells in the human supplements compared to FBS grown colonies [13]. From the proliferation studies such as growth kinetics, population doubling time, cell proliferation using BrdU and CFU assay, it was evident that 2.5% of PL supplemented medium was sufficient for the expansion of MSCs in an optimal way.

The typical characteristic feature that distinguishes an MSC is the expression of specific surface antigen. MSCs at various percentages of PL were assessed for the expression of the surface antigens (CD 14, 34, 45, 73, 90 and 105). The MSCs did not exhibit any change in the surface antigen ex-

pression levels both for positive (CD 73, 90, 105) and negative (CD 14, 34, 45) surface antigen markers irrespective of the varying concentration of PL.

Another characteristic feature that defines MSCs is their trilineage differentiation potential which makes them suitable for repair and rejuvenation of diseased and ageing tissues. Hence understanding the differentiation potential of MSCs in the context of its expansion using varying concentrations of PL would favour its role in the clinical setup. Therefore, we have attempted to delineate the trilineage differentiation capacity of MSCs expanded and differentiated at all concentrations of PL supplemented medium. Mineralized bone matrix, a hallmark of the final phase of osteogenic differentiation, is tightly regulated by the expression of osteogenesis-related genes osteocalcin (OCN), Collagen type 1A (Col 1A) [22]. In the qualitative osteogenic differentiation analysis, we observed that the mineralization started earlier *i.e*., day 7 in the MSCs differentiated at higher concentrations of PL (7.5% and 10%) compared to 2.5% and 5% which exhibited mineralization at 10 and 12 days. But for cells differentiated at 10% FBS, mineralization was observed at day 14. Hence it can be inferred that PL has profound effect on the osteogenic differentiation even at a lower concentration compared to 10% FBS. Gene expression studies for collagen type 1A and osteocalcin which are the early and late osteogenic differentiation markers also confirmed the above. During the process of osteogenic differentiation, collagen type 1A is expressed early to form the matrix which supports mineralization followed by the expression of late markers osteocalcin and osteopontin, which aid in the calcium and phosphate deposition [23]. Upregulation of both collagen type 1A and osteocalcin was observed during osteogenic differentiation of MSC performed at all the concentrations of PL. Therefore, 2.5% of PL is sufficient to maintain the osteogenic differentiation ability of MSCs.

Adipogenic differentiation was observed by oil red O staining. We observed that adipogenic differentiation was initiated earlier for MSCs cultured at 10% PL which was on par with 10% FBS (day 12). Whereas, for 7.5%, 5%, 2.5% of PL it was at day 14, 16, 16 respectively. In the gene expression study for the adipogenic genes such as adiponectin, PPARγ and RunX2, it was observed that maximum expression of PPARγ genes was at 2.5% PL itself, adiponectin at 7.5% PL and RunX2 at 5% PL. However, at other concentrations of PL also exhibited adequate expression of adipogenic genes implying that all concentrations of PL could support adipogenic differentiation.

In the chondrogenic differentiation study by toluidine blue staining, differentiation was observed at day 21 wherein the number of chondrocyte micromass formed was high at 2.5% PL (4 micromass pellet) on par with 10% FBS (5 micromass pellet). Whereas the other concentrations of PL 5%, 7.5% and 10% showed 2, 2 and 3 micromass pellet respectively. These observations could be correlated by chondrogenic differentiation gene Sox 9 which was expressed at all the concentrations of PL.

The role of PL supplementation in the growth medium relating to the stemness properties of MSCs was assessed. It is inferred that PL at all the concentration was able to express the stemness genes Oct4, Nanog and Sox 2. In fact, significant up regulation in expression of all the genes at all concentrations of PL was comparable to that of 10% FBS. Therefore, it is clearly evident that PL supplementation maintained the pluripotent stemness property of MSCs.

For the use of MSCs in clinical transplantation, assessment of chromosomal stability is mandatory. Recent evidences have shown the presence of chromosomal abnormalities in GMP grade MSCs by cytogenetic studies. Evidence of clonal mutations was also seen but not associated with *in vitro* malignant transformation [24, 25]. This necessitated us to ensure the genetic stability of MSCs cultured at the different concentration of PL before its use in clinical application. Cell karyotyping is a reliable indicator for evaluating genetic stability. In the present study, MSCs cultured at different concentrations of PL (2.5%, 5%, 7.5% and 10%) and 10% FBS were assessed for chromosomal stability during subsequent passages. Normal diploid karyotypes (*i.e*., 46 XY without any aneuploidy or polyploidy) was seen for MSCs expanded using PL from passage 2 -10. Deletion, inversion, translocation nor ring chromosomes were seen in any of the passages involved. Thus, our present study confirms the stability of chromosomes in both PL and FBS supplemented media.

To avoid the risk of infection during clinical transplantation, MSC cultures must be free of microbial contaminants. In the study, all MSCs cultured were analyzed and found to be free of mycoplasma and endotoxin.

CONCLUSION

This study demonstrates that hPL can be used as an alternative for FBS in the culture and expansion of MSC. We have demonstrated that 2.5% of PL supplementation in the culture medium was sufficient to culture, expand and preserve the phenotypic characteristics of MSCs such as the surface antigen levels, colony formation, differentiation into mesodermal lineages and stemness properties. Also, at this concentration of PL, the MSCs exhibited maximum proliferation by shortening their doubling time, major criteria to be considered during cell therapy. Hence, we infer that the components present in the 2.5%PL were comparable to or higher than 10% FBS. Likewise, single donor derived PL at 2.5% concentration also exhibited similar effects during expansion and differentiation of MSCs indicating the advantages of using autologous PL or donor recipient matched PL for clinical application. Thus, PL medium proved to be a safer medium which is easy to obtain from the platelet concentrate and has the advantage of being an autologous humanised substitute to avoid immune rejection for the production of clinical grade MSCs.

LIST OF ABBREVIATIONS

7-AAD	=	7-Amino-Actinomycin D
BRDU	=	5-Bromo-2-Deoxyuridine
CFU	=	Colony Forming Unit
COL1A	=	Collagen Type 1A
DMEM	=	Dulbecco's Modified Eagles Medium
FBS	=	Fetal bovine Serum

ISCT	=	International Society for Cell Therapy
ISCT	=	International Society for Cell Therapy
KCL	=	Pottassium Chloride
LAL	=	Limulus Amoebocyte Lysate
MSCs	=	Mesenchymal Stem Cells
OCN	=	Osteocalcin
PC	=	platelet Concentrates
PCR	=	Polymerase Chain Reactions
PDT	=	Population Doubling Time
PL	=	Platelet Lysate
PRP	=	Platelet Rich Plasma
qRT-PCR	=	Quantitative Real Time PCR
TPRP	=	Thrombin Activated Platelet Rich Plasma (Tprp)
WHO	=	World Health Organisation
WJ-MSCS	=	Whartons Jelly Derived MSCs

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of Government Stanley Medical College, Chennai, India (ECR/131/Inst/TN/2013/RR-16 dated 17/02/2017) and Institutional Committee for Stem Cell Research of Government Stanley Medical Hospital, Chennai (Registration No NAC-SCRT/79/20152002)/Proposal no 02/2016.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. All human procedures were performed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Informed consent was obtained from the mothers of patients involved in the study.

AVAILABILITY OF DATA AND MATERIALS

Most of the data generated/ analyzed during this study are included in this Published article. The datasets of the current study which are not shown in this article are available from corresponding author on reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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