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Research Paper

COMPARATIVE EFFICACY OF FICOLL HYPAQUE AND HYDROXYETHYL STARCH PROTOCOLS FOR THE ISOLATION OF HUMAN BONE MARROW DERIVED MESENCHYMAL STEM CELLS

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Abstract

Stem cells differ from other kinds of cells in the body. All stem cells regardless of their source are unspecialized, capable of dividing and renewing themselves for long periods and can give rise to specialized cell types. It serves as a sort of repair system for the body. They can theoretically divide without limit to replenish other cells as long as the person or animal is alive. This promising area of science has led scientists to investigate the possibility of cell based therapies to treat diseases which are often referred to as "regenerative or reparative medicine". In the present study, human bone marrow sample which serves as the primary source for Mesenchymal stem cells (MSCs) was used. MSCs from the bone marrow aspirate were isolated using ficoll hypaque (1.077g/mL) and 6% Hydroxylethyl starch (HES). Ficoll hypaque is currently the leading density gradient medium for the preparation of human mononuclear cells from small volumes of blood. When cell count was performed after the isolation protocol, samples subjected to ficoll hypaque had low cell count and erythrocyte contamination was also low. On the other hand, HES, a density gradient medium, is used for isolation of MSCs from relatively large volumes of blood samples using sedimentation technique. However, erythrocyte contamination was a major problem during this isolation protocol. Samples treated with HES showed high cell count and also the percentage of erythrocyte contamination was more, which led to overcrowding of cells in the culture flasks when seeded. Also, per cent cell viability was more when ficoll hypaque was used for isolation of MSCs. On the fourteenth day of culture, the culture flasks were taken and Colony forming unit (CFU) assay was performed which gave the number of MSCs present, in the total number of cells seeded. It was found out that samples treated with Ficoll hypaque yielded more colony forming units when compared to those treated with HES. Erythrocyte contamination probably could be the

reason for the formation of less number of colony forming units in culture when HES was used as the isolating medium. This shows that amongst the two methods, Ficoll hypaque may be considered as the apt medium for the isolation of MSCs when compared to HES due to less erythrocyte contamination.

Key words: Mesenchymal stem cells, ficoll hypaque, hydroxylethyl starch, colony forming unit, population doubling time.

INTRODUCTION

Mesenchymal stem cells (MSCs) are non-hematopoietic cells, which reside in the bone marrow together with better known and characterized class of stem cells called hematopoietic stem cells [1]. They were first described by Friedenstein *et al.* [2] as fibroblast precursors from bone marrow. They are called MSCs, because of their ability to differentiate into mesenchymal-type cells *viz*., osteoblasts, chondrocytes, myocytes, adipocytes, cardiomyocytes, neurons [3] and beta pancreatic cells [4]. MSCs are also referred to as fibroblast colony-forming units (CFU-Fs) or marrow stromal cells [5], since they appear to arise from the complex array of supporting structures found in the marrow [6]. The main source of MSCs is bone marrow [7] and is also located in other tissues of the human body. There are a number of reports describing their presence in peripheral blood [8], adipose tissue [9], foetal liver [10], umbilical cord blood and lungs [11], exfoliated deciduous teeth [12], chorionic villi of the placenta [13] and amniotic fluid [14]. MSCs have been studied extensively because of their high potential for multi-lineage differentiation and proliferation characteristics that make the cells useful for therapeutic purposes [7]. Another notable characteristic is plastic adherent ability of MSCs when cultured in appropriate media [15].

MSCs retain their multipotency even after some passages [16]. The characteristics of MSCs from fresh bone marrow may change during long-term culture, like genotypic change, phenotypic instability, transformation, loss of differentiation capacity and decrease of proliferation ability or senescence [17]. MSCs are positive for CD105, CD73 and CD90 and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR [18]. The identification of a definitive marker allowing the prospective isolation of MSCs from fresh tissue would be of utmost importance [19]. The present study compares the efficacy of two methods *viz*., Ficoll hypaque and Hydroxyl ethyl starch (HES) used for the separation of MSCs from bone marrow and also focuses on the expansion and characterisation of MSCs using cell surface markers.

MATERIALS AND METHODS

ISOLATION OF MSCS USING FICOLL HYPAQUE AND HES

The bone marrow samples (ten) (5mL) obtained from Frontier Life Line Hospitals, Mogappair, Chennai, Tamil Nadu, India were carefully overlaid onto the ficoll gradient and centrifuged at 1500rpm for 30 minutes. Three distinct layers were observed with the topmost layer containing plasma, the central buffy coat which is the layer of mononuclear cells (MNCs) and the bottom layer with both erythrocytes and leucocytes. The mononuclear cell layer was carefully transferred into a centrifuge tube and was then centrifuged at 1500rpm for 10 minutes. The pellet obtained was washed with phosphate buffered saline (PBS) and centrifuged at 1500rpm for 10 minutes. The supernatant was discarded and the pellet was again resuspended with PBS and centrifuged at 1500rpm for 10 minutes. The final pellet obtained was used for cell seeding. Approximately 5 x 10^5 to 1 x 10^6 cells/25cm^2 was seeded into a culture flask

containing Dulbecco's Modified Eagle's Medium (DMEM) with 10% Foetal bovine serum (FBS) and 1% antibiotics of penicillin, streptomycin and ampicillin. The culture was then maintained in 5% carbon dioxide incubator.

In the case of isolation of MSCs using HES, the bone marrow sample and 6% HES were taken in a centrifuge tube in the ratio 1:3 and agitated for 20 minutes. The tube was kept for sedimentation for 1 hour. The supernatant was then carefully transferred into a centrifuge tube and centrifuged at 1500rpm for 10 minutes. Thereafter, the same methodology was adopted for isolation of MSC using ficoll hypaque mentioned elsewhere was followed.

CELL VIABILITY COUNT

The cell fraction isolated from the above protocols was subjected to cell counting and viability tests. The viability was estimated using trypan blue exclusion method. The cells which were viable were generally able to exclude the dye and appeared clear against a blue background of the trypan dye. The dead cells were not able to exclude the dye and appeared dark blue with stain inside the cell. It is often essential to know the proportion of viable cells in a cell suspension. Neubauer chamber was cleaned with alcohol, dried and wiped with a clean muslin cloth. The cover slip was then mounted on the counting chamber. To the cell suspension (10µL), trypan blue dye solution (10µL of 0.4%) was added and mixed. The stained suspension (10µL) was placed at the edge of the cover slip and the counting chamber was filled. Excess fluid was avoided. All the unstained cells (viable cells) and stained cells (dead cells) in $1mm^2$ were counted. The per cent viability of the cell suspension was calculated using the formula.

$$\text{Per cent viability} = \frac{\text{Number of living cells}}{\text{Total number of cells}} \times 100$$

COLONY-FORMING UNITS (CFU) ASSAY

MNC from donors were seeded and incubated for 14 days in DMEM without medium exchange under a humidified atmosphere and thereafter, the cell suspension was removed. The adherent cells were fixed with methanol, dried and stained for 5-10 minutes with crystal violet. After washing with running tap water, the plates were observed for the presence of colony forming unit fibroblasts (CFU-F), which were stained with the crystal violet.

SUB-CULTURING (PASSAGE)

The cells on becoming 70% confluent, was checked for cell density, morphology and contamination if any with aid of an inverted microscope. The monolayer was washed twice with PBS. The PBS was allowed to stand on the cells for 30 seconds to remove as much extracellular proteins as possible. The PBS was discarded and the adherent cells were then removed by adding 4mL of Trypsin-Ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin and 0.02% EDTA). Typsinisation was carried out for 10 minutes at 37˚C to remove the adherent cells. PBS was added and the cells were gently scrapped. The cell suspension was centrifuged at 1500rpm for 10 minutes and the pellet was collected. The pellet was resuspended in PBS and centrifuged at 1500rpm for 10 minutes to remove traces of trypsin. The above step was repeated once more. The cells were then seeded into a new T-25 flask at a final concentration of ~2 x 10^5cells/mL.

IMMUNOCYTOCHEMISTRY

The immunocytochemical staining method uses one antibody against the antigen being probed for, and a secondary antibody against the first. Initially, the cells were rinsed twice with PBS followed by removal of the liquid by gentle aspiration. The cells were then fixed with 4% paraformaldehyde in PBS for about 10-20 minutes at room temperature and then briefly rinsed twice with PBS. Fixed cells were then permeabilised with 0.2% Triton X-100 in PBS for 5-10 minutes. Cells were washed thrice with PBS and were incubated with PBS containing 5% Bovine Serum Albumin (BSA), which is a blocking agent, overnight at 4˚C. Primary mouse monoclonal antibody of CD44 diluted in PBS containing 1% BSA (1:400) was added to the cell culture and incubated for 3-4 hours at 4˚C. Cells were then washed thrice with PBS and twice with PBS containing 1% BSA. Antimouse secondary antibody (1:200), fluorophore labelled, diluted in PBS containing 1% BSA was now added to the same culture and incubated overnight at 4˚C. Cells were then viewed under fluorescent microscope to detect the presence of CD44 cell surface marker. The same procedure was followed for cell surface markers CD73, CD90 and CD105. The fluorophores used were Fluorescein isothiocyanate (FITC) for CD44 and CD90; and Phycoerythrin for CD73 and CD105.

POPULATION DOUBLING ASSAY (PDA)

On reaching confluence, cells were passaged using Trypsin-EDTA solution. The passaged cells were then plated equally in each well of the six well plate. Cell count was performed before seeding. After 48 hours, the cells from any one well of the six well plate was taken after trypsinisation and cell count was performed. Subsequently the cells from the other wells were also taken after trypsinisation for cell count i.e. each well after 24 hours interval. Population doubling time (PDT) was then calculated using the formula, PDT = Log (Number of cells at harvest) – Log (Number of cells at seed). A graph was then plotted to depict the PDT.

RESULTS

A dense band of mononuclear cells was harvested using ficoll hypaque technique and mononuclear cells contaminated with erythrocytes were collected using HES method. The harvested MSCs, after comparative study of two isolation techniques, were cultivated and analysed for their morphological characteristics. Bone marrow derived MNCs were round shaped when seeded. They formed adherent heterogeneous cell populations after day 3 in culture consisting of spindle-shaped cells. They underwent extensive proliferation between days 5 and 9, with colony formation around days 10 to 14 (Figure 1). The cell population reached confluence within three weeks of culture. Total cell count and cell viability count was performed after both the isolation protocols. Samples subjected to ficoll hypaque had low total cell count due to less or no erythrocyte contamination. Subsequently, cell viability count was also low and the percent cell viability was found to be 94.95%. Samples treated with HES showed high total cell count because of high percentage of erythrocyte contamination. The cell viability count for HES samples was lower than the total cell count and the per cent cell viability was found to be 82.48% (Figure 2A, 2B, 2C). Samples treated with ficoll gradient yielded more colony forming units in culture whereas those treated with HES yielded less number of colony forming units in culture (Figure 2D, 3). Immunocytochemistry performed gave positive results for the markers CD 44, CD 73, CD 90 and CD 105, thereby proving that the cultured cells were MSCs (Figure 4). PDA

showed a gradual increase in the growth rate and population doubling time was 66.31 hours (Figure 5).

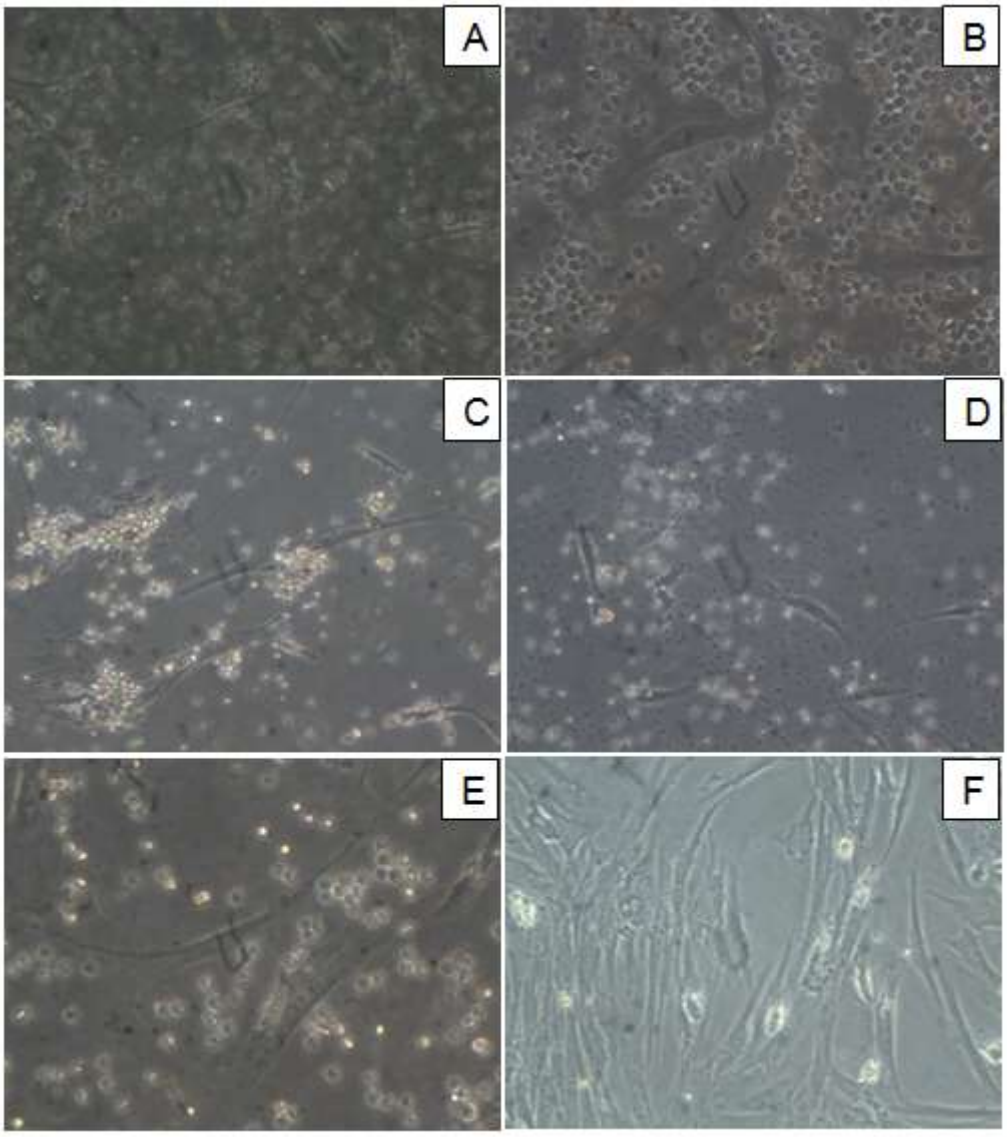

Figure 1. Morphological characteristics of MSCs
A: Early spindle shaped cells; B: Day 6; C: Day 7; D: Day 8; E: Day 9; F: Day 14

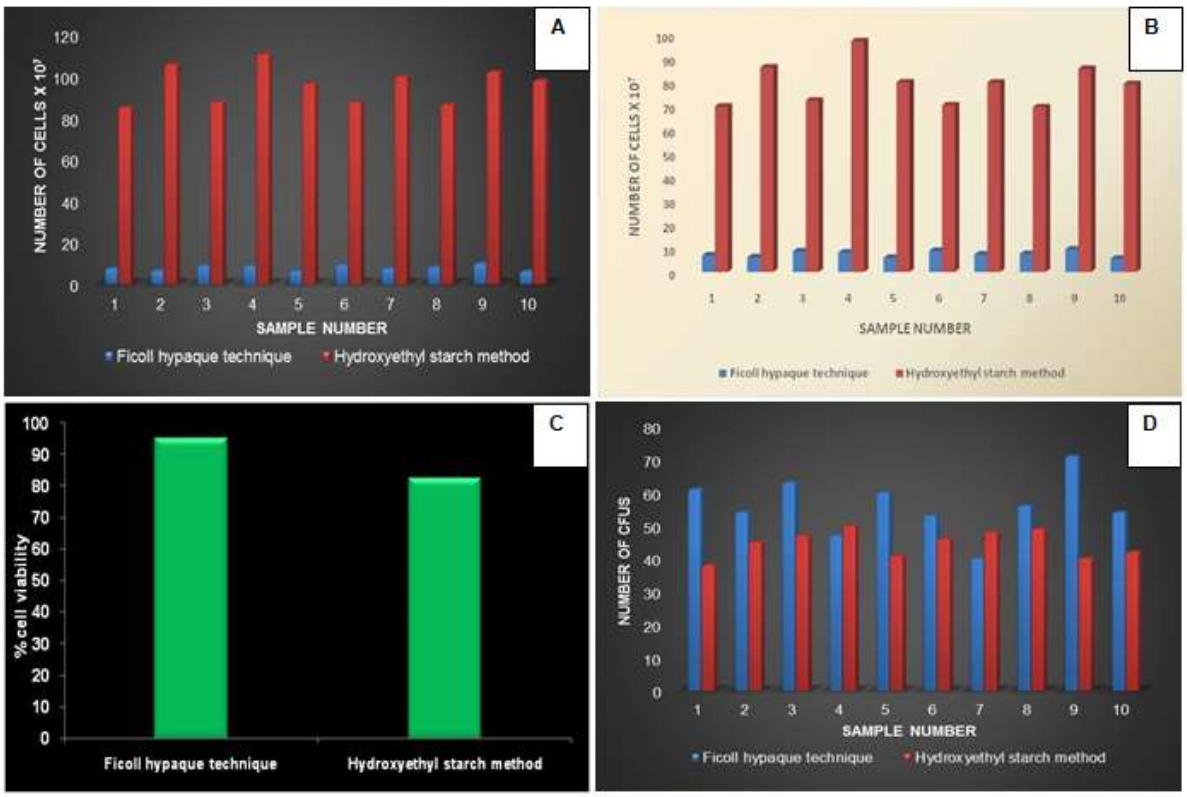

Figure 2. Comparative efficacy of ficoll hypaque technique and hydroxyethyl starch method
A: Total cell count; B: Cell viability count; C: Per cent cell viability; D: Number of CFUs

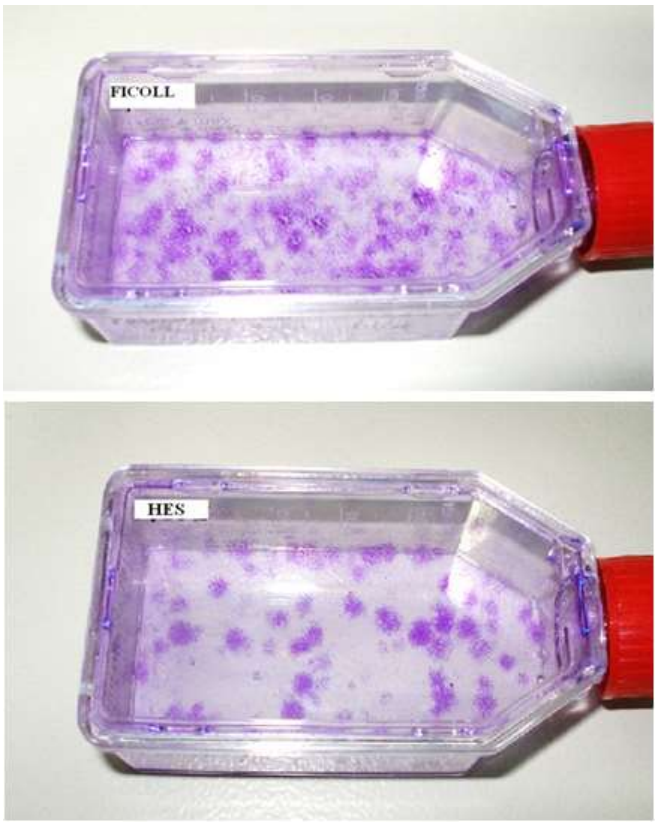

Figure 3. CFU assay

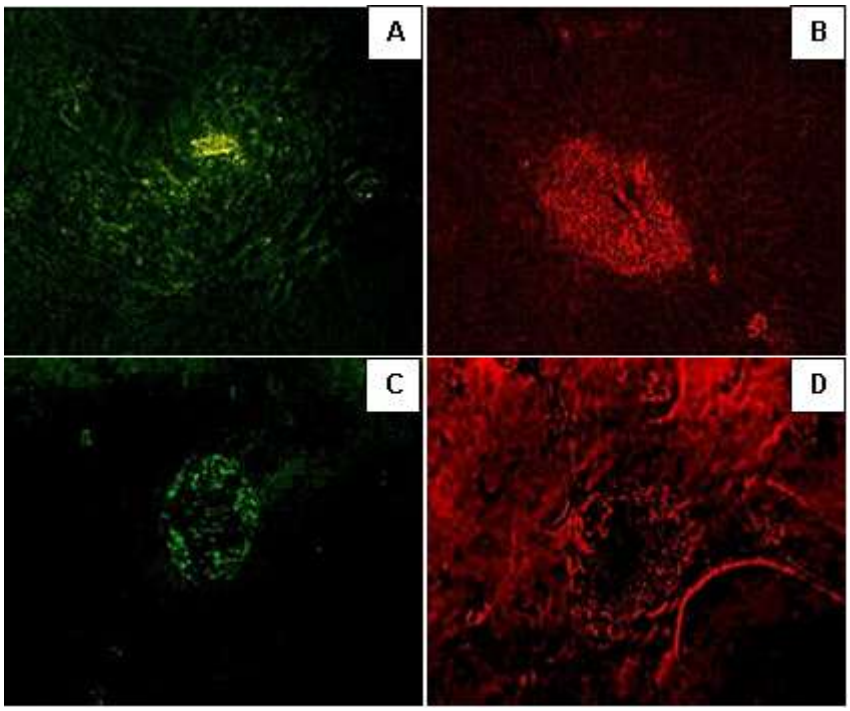

Figure 4. Immunocytochemistry
A: CD44; B: CD73; C: CD90; D: CD105

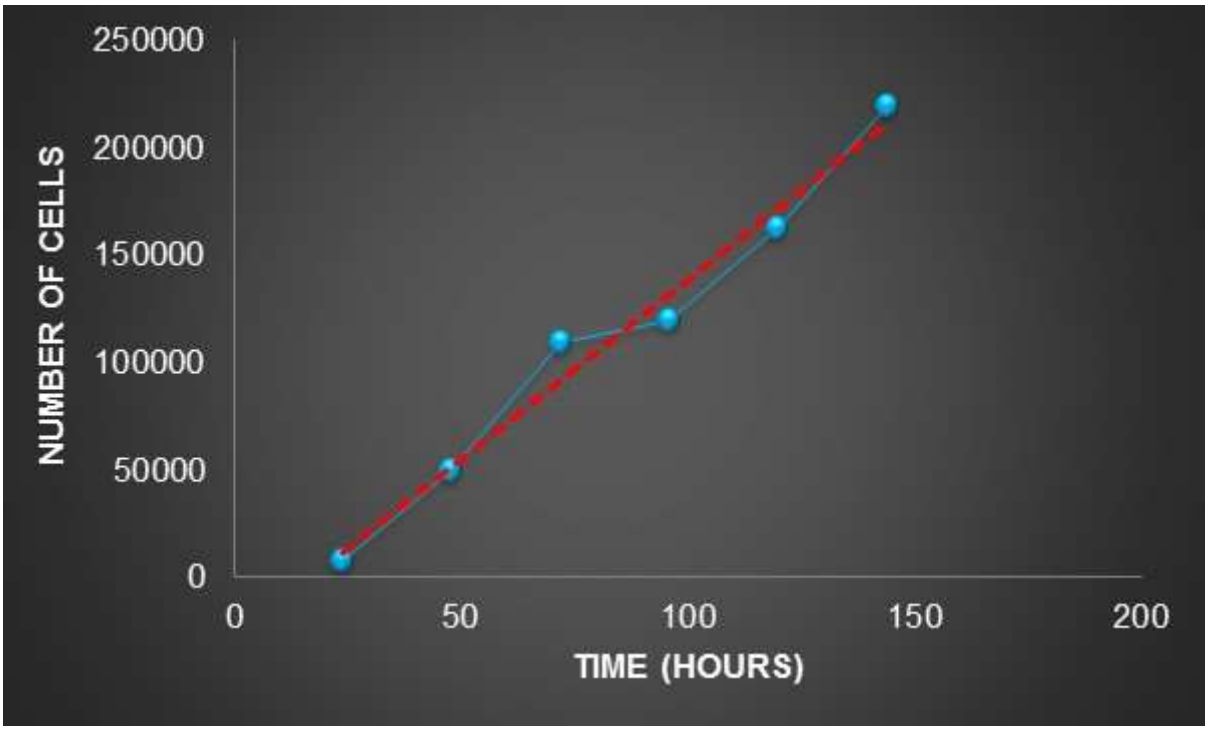

Figure 5. PDT

DISCUSSION

Ficoll hypaque is a sterile ready to use density gradient medium for the preparation of mononuclear cells in high yield and purity, from small volumes of blood samples using a simple and rapid centrifugation procedure [20]. Several factors contribute to the success of this separation. Ficoll gradient centrifugation has a specially designed tube with two

chambers separated by a porous high density polyethylene barrier or frit. The anticoagulated blood when added to the top chamber of the tube without risk of mixing with the histopaque, the whole blood descends through the frit to contact with the histopaque during centrifugation. The elements of greater density displace a volume of histopaque above the frit giving a clear separation of the blood components. The erythrocytes aggregate and the granulocytes become slightly hypertonic, increasing their sedimentation rate, resulting in pelleting at the bottom of the tube. Lymphocytes and other mononuclear cells, i.e., monocytes, remain at the plasma-histopaque interface [21]. This dense band of mononuclear cells contains MSCs and is of importance. Erythrocyte contamination is thus avoided due to the barrier between the chambers. Therefore, when cell count was performed after the isolation protocol, samples subjected to ficoll hypaque had less total cell count and erythrocyte contamination was lower. It was also found out that samples treated with ficoll gradient yielded more CFUs in culture owing to high percent cell viability [22].

HES, a density gradient medium, can also be used for isolation of MSCs from relatively large volumes of blood samples using a sedimentation technique [23]. Erythrocyte contamination was a major problem during this isolation protocol. When cell count was performed after isolation, samples treated with HES showed high cell count due to erythrocyte contamination, which led to the overcrowding of cells in the culture flasks when seeded. Also, the percent cell viability was lower when compared to Ficoll hypaque method. This probably could be the reason for the formation of less number of colony forming units in culture. Therefore in comparison, ficoll hypaque may be considered as the best medium for the isolation of MSCs from bone marrow [24] when compared to HES due to less erythrocyte contamination. MSCs showed a gradual increase in the growth rate [25] when PDA was performed and population doubling time was 66.31 hours. CD44, CD73, CD90 and CD105 cell surface markers were found in the undifferentiated form of MSCs which makes it possible to separate the earliest precursors of MSCs from more mature cells [26]. CD44 is strongly expressed and is a receptor for various ligands like hyaluronan and osteopontin, which plays a central role in the organization of the extracellular matrix in the marrow or in the bone respectively. The most discriminative marker for MSCs examined after short time of adherence (1-3 days) was CD73 and was extensively expressed. CD90 and CD105 were less pronounced [27]. This study therefore confirms that, Ficoll hypaque technique may be considered as the apt method to isolate human bone marrow derived MSCs. The two isolation protocols discussed in the study will be useful to isolate MSCs from other sources as well.

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