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Isolation and Immunophenotypic Characterization of Bone Marrow Derived Stem Cells

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ABSTRACT : *Human bone marrow derived Mesenchymal stem cells (HBM-MSCs)non-haematopoietic, that can differentiate into a number of cells types namely adipocytes, osteocytes, chondrocytes, cardiomyocytes, myocytes etc., and represent a promising cell-based therapy for a number of degenerative conditions. In the present study, the bone marrow aspirate was taken and the MSCs were isolated using ficoilhypaque protocols. The presence of surface markers (CD34+, CD45+, HLA-DR+, CD73+, CD44+, CD13+, CD90+, CD105+) was analyzed by immunocytochemistry.*

KEY WORD: *Adult stem cell, Flowcytometry, PBS, ficoll, Hemocytometer, Surface Markers, Mesenchymal stem cells.*

I. INTRODUCTION:

Stem cell research has undergone huge advances in the past couple of years. It has proved particularly challenging for scientists to ensure the long term proliferative ability and pluripotency of embryonic stem and germ cells. Bone marrow is the flexible tissue in the interior of bones. In humans, red blood cells are produced by cores of bone marrow in the heads of long bones in a process known as hematopoiesis. On average, bone marrow constitutes 4% of the total body mass of humans; in an adult weighing 65 kilograms (143 lb), bone marrow typically accounts for approximately 2.6 kilograms (5.7 lb). The hematopoietic component of bone marrow produces approximately 500 billion blood cells per day, which use the bone marrow vasculature as a conduit to the body's systemic circulation (Vunjak Novakovic *et al,*. 2010). Bone marrow is also a key component of the lymphatic system, producing the lymphocytes that support the body's immune system.

Bone marrow transplants can be conducted to treat severe diseases of the bone marrow, including certain forms of cancer. Additionally, bone marrow stem cells have been successfully transformed into functional neural cells, and can also potentially be used to treat illnesses such as inflammatory bowel disease. The bone marrow stroma contains mesenchymal stem cells (MSCs) (Raphael Rubin *et al,*. 2007), also known as marrow stromal cells. These are multipotent stem cells that can differentiate into a variety of cell types. MSCs have been shown to differentiate, in vitro or in vivo, into osteoblasts, chondrocytes, myocytes, adipocytes and beta-pancreatic islets cells.Immune challenges also prove a significant barrier to the application of cell therapies. If the stem cells are recognized as non-self, they will be rejected and destroyed. Two potential solutions to this problem have been proposed. One solution in the creation of universal donor stem cells through genetic engineering techniques. Theoretically stem cells could be created that lack outer surface labels. The absence of these labels, which normally identify cells as non-self, would eliminate the problem of immune rejection would prove less labour intensive and more cost effective, than the latter solution. Adult stem cell treatments have been successfully used for many years to treat leukemia and related bone/blood cancers through bone marrow transplants. Adult stem cells are also used in veterinary medicine to treat tendon and ligament injuries in horses (Kane, Ed *et al,*. 2008). The therapy through cell transplant has been developed based on the adult multipotent stem cell is becoming, consequently, an important scientific subject (Ana Maria *et al,*. 2008). It has already been proved that most promising stem cell source in clinical practice is represented by bone marrow with more focus on candidate stem cells especially HSC and MSC(Ana Maria *et al,*. 2008).

However, recent development in stem cell biology has demonstrated the presence of sub population cells especially side population (SP) cells endothelial progenitor cells along with stem cells which is said to possess the properties of repair and regeneration (Challen GA *et al,*. 2006, Margaret *et al,*. 1996, Carmen *et al,*. 2006). Asahara and colleagues published that purified CD 34+ hematopoietic progenitor cells from adults can differentiate ex vivo to an endothelial phenotype (Asahara *et al,*. 1996,). Moreover, focus on homogenous candidate stem cells by sorting of CD 34+ HSC by FACS (Loken *et al,*. 1996), lineage depletion by MACS (Dhahlke MH *et al,*. 2002, Forraz N, *et al,*. 2004, Granthos *et al,*.2008) ,and expansion of MSC in culture gained importance in cell therapy.

Adult stem cells hold great promise in the field of regenerative medicine. Among all types of adult stem cells, mononuclear cells obtained from bone marrow using density gradient method are widely used as a potent source of clinical application (Serban *et al*., 2008; Kondo *et al*., 2003; Kumar *et al*., 2009).This is because of their existence as immature cells in the niche that has high proliferative and differentiation potency unlike peripheral blood MNC (George *et al*., 2005; Witson *et al*., 2006; Jansen *et al*., 2007). A Major limitation of current Mononuclear cell therapy is the low "Payload" of therapeutic stem cells delivered to the site of injury, thus being an obstacle for curing diseases (Leblond *et al*., 2009;Scherscher and Soonpaa,2008; Murry and soonpaa,2004). This limitation is because of the granulocyte interface and debris along with mononuclear cells and less plasticity (Kucia *et al*., 2005).Thus, finding an alternative approach of obtaining more percentage of stem cells from cost effective procedure without any debris is of utmost importance. Several researchers have worked on the FACS and MACS based cell purification method (Broers *et al*., 2003; Pruszak *et al.*, 2007; Herzenberg *et al*., 2002; Malatesta *et al*., 2000).

II. MATERIALS AND METHODS:

COLLECTION OF BONE MARROW:

Bone marrow was collected after the approval from the ethical committee and the concern of donors. Stem cells are aspirated from iliac crest of patient or donor using jamshidi needle. The needle was carefully injected into centre potion of bone. The blood was aspirated and processed further. Generally these bone marrow stem cells were collected form back side of body from pelvic region. This region was selected because it was considered as the biggest bone of body and can be isolated easily.

ISOLATION OF MONONUCLEAR CELLS FROM BONE MARROW PROCESSING:

Bone marrow sample was taken and heparin was added to it to prevent coagulation and was mixed well. The sample was dissolved in PBS in 1:2ratio. In a sterile conical bottom test tube, ficoll gradient solution and sample were taken in 1:3ratio. The diluted blood was layered on ficoll gradient solution. Centrifugation was done at 400g for about half an hour at 21°C with zero acceleration and deacceleration. Now, distinct layers will be formed such that mononuclear cells will form a this white layer just above the gradient solution First the plasma was pipette out, than the white layer containing the required mononuclear cells was removed carefully and added to a 50ml of conical bottom tube. PBS was added to it and centrifugation was done at 450g for ten minutes. To the pellet 0.7% of ammonium chloride of P^H 7.4 to lyses to RBC's present if any. And left for about 2 minutes and 0.9% sodium chloride was added at 4°C to stop the activity of ammonium chloride. Centrifugation was done for 300g about 10 minutes at 4°C to remove the lyses RBC's. Isolated lymphocytes were ready for characterization and further analysis and processing.

CELL COUNTING:

The hemocytometer chamber was properly cleaned before performing the test. The cover sip was mounted on the chamber such as it covers the grid lines on both sides. The sample is diluted at 1:10 dilution i.e., 10 µl of sample and 90 µl WBC diluting fluid. After mixing it properly 10 µl of suspension was taken in a pipette. The pipette is brought near the neubauer chamber ad pressed so that it forms a drop and due to capillary action the drop gets sucked into the chamber. This setup was viewed under microscope and was first viewed under10X objectives to focus the image properly. This is then changed to 40X objective to count the total number of cells. The four corners of the hemocytometer were viewed under40x objective to count the total number of cells present in the sample. Hence both the viable and non-viable cells in the chamber were counted to calculate the total cell count in the suspension. The concentration of cells = Number of cells x Dilution factor x (depth factor/ number of squares)

CELL VIABILITY TESTING:

The hemocytometer chamber was properly cleaned before performing the test. The cover slip was mounted on the chamber such as it covers the grid lines on both sides. The sample is diluted at 1:10 dilution i.e., 10 µl of sample and 50 µl of PBS and 45 µl of 0.4% trypan blue dye. After mixing it properly 10 µl of suspension was taken in a pipette and the pipette is brought near the neubauer chamber and pressed so that it forms a drop and due to capillary action the drop gets sucked into the chamber. This setup was viewed under microscope and was first viewed under10X objectives to focus the image properly. This is then changed to 40X objective to count the total number of cells. The four corners of the haemocytometer were viewed under40X objective to count the total number of cells present in the sample. Hence, both the viable cells in the chamber were counted to calculate the % viability of the suspension. The % viability of cell suspension was calculated. Percentage of viability = (number of viable cells/total ells) X100.

CHARACTERIZATION OF BONE MARROW CELLS USING FLOWCYTOMETRY

From bone marrow $1x10^6$ cells samples were taken and characterized for various hematopoietic, mesenchymal and side population markers using BD FACS Aria. The CD 34 Hematopoietic population, the CD45 mesenchymal marker and the side population cells were used for characterization in Flowcytometry.

FLOW CYTOMETRIC PROTOCOL FOR CHARACTERISATION

Flowcytometry was performed on a Beeton, Dickinson FASC Aria using a 488nm agron-iron LASER and 632nm red las-LASER for excitation; fluorescence emission was collected using its corresponding detectors. $1x10^6$ cells were stained with appropriate amount of conjugated antibiotic in each of 12x75mm falcon polystyrene FACS tube, BD Bioscience, cat no;352054. The quality of each antibody conjugated with flluorochromes added to the cells in each tube were 20ul of CD34, CD45, 20ul of CD34-PE, 20ul of CD45-FIPC, 20ul 7 AAD-per-CP C45 20ul CD90-per CP C45, CD-105-APS 20ul of CD73-PE, 20ul of CD29-per, HCADR-per CP, CD 117-APC respectively. All tubes are incubated for 20 minutes in darks. After incubation, cells were washed in phosphate buffer saline to remove the unbound antibodies. The pellet was further suspended to 500ul. Data analysis and acquisition was then performed using DIVA software. Flowcytometry instrument were set using unstained cells. Cells were gated by forward versus side scatter to eliminate debris. The number of cells staining positive for a given markers was determined by the percentage of cells present within a gate established. A minimum of 10,000 events was characterized and recorded.

III. RESULT

The mononuclear cells of bone marrow are our main concern as they remain undifferentiated in the niche. Maturation and differentiation begins once the mobilize from bone marrow and reach their respective site of differentiation begins once they mobilize from bone marrow and reach their respective site of differentiation, thus mononuclear cells are important. Fluorescent Activated Cell Slotter analysis by the use of CD markers revealed the presence of various stem cells constituting the bone marrow. Here, table-1 tabulates the percentage or quantitative estimation of different stem cells.

Interpretation of (**Table-1 and Graph-1)** confirmed that availability of hematopoietic stem cells, mesenchymal stem cells and endothelial cells in parent population. Endothelial cells were contribute to highest number (in parent population) followed be mesenchymal stem cells and then hematopoietic stem cells. To study the characterization of bone marrow derived stem cells using different markers with FACS at the stage has MNC populations which were marked with CD34+, CD45+, HLA-DR+, CD73+, CD44+, CD13+, CD90+, CD105+has showed the best results. MNC populations showed 2.8%, 89.0%, 30.0%, 81.3%, 94.2%, 37.1%, 0.3%, and 0.2% respective to the markers used (**Table: 1 & Graph:1**).The amount of mesenchymal stem cells extracted is not limited. The present study showed that to extract a good percentage of mesenchymal stem cell population. It was inferred from Fluorescent Activated Cell Slotter result column.

IV. TOTAL CELL COUNT:

Sample 20ml of bone marrow blood was processed using ficoll and the final pellet was resuspended with PBS to 2ml.The total count obtained on 4 square was 21, 24, 32, and 25. Hence totally 102 bone marrow blood cells was analyzed by applying the formula for total WBC counting;

Total volume of blood processed = 20ml.

Total volume of pellet mad up to 2ml

Total count = Number of cells x Dilution factor x (depth factor/ number of squares).

= 102x2x10/4

= 510, Hence 51 x 10^5 cells / µl

Therefore, $5.1x10^5$/ml

= $10.2x10^6$ / 2ml

VIABILITY:

Percentage of viability = (number of cells / total cells) x 100.

= (99/102) x 100

= 0.970 x 100

= 97%

Interpretation of (**Graph-2)** explained around 97% of cells were viable remaining 3% of cells are dead cells.

EFFICIENCY OF PROCESSING OF MONONUCLEAR CELLS:

The present study revealed that **(Table-2 and Bar Diagram-3)** explained the efficiency of the before and after processing of MNC populations which was marked with Lymphocytes (32.92%, 65.20%), Monocytes (3.70%, 3.70%), Granulocytes (40.50%, 0.20%) has showed. When the processing was not efficient and the

buffy coat layer was not isolated properly and the granulocyte percentage was observed just 0.2 percentages. This result suggested that how efficient was the processing. Ensuring this step was very important as granulocyte causes injury or inflammation and interferes with the plasticity of stem cells.

V. DISCUSSION

Bone marrow was composed of heterogeneous population. Hence, a single aspiration could be used to treat different diseases. The availability of all three stem cells was convincing quantity wise. This amount to approve the assured the presence of stem cell regardless to the quantity of bone marrow was aspirated. It has already been proved that bone marrow was the promising source of stem cell therapy with presence of HSC and MSC (Challen, *et al.,* 2006). The Efficiency of the processed mononuclear cells from bone marrow was importance before cellular characterization or cellular transplant. The present study showed that to confirm whether processed bone marrow MNC (n=5) were devoid of contaminants and it was highly efficient for further research and therapy. The present research performed a differential count analysis before and after MNC processing **(Figure 1, Table 1).**Though granulocytes live only ~10hrs in circulation, there exist many complications of granulocyte interface with MNC. It might even disrupt the stem cell from further engraftment. Hence, this research emphasize on the fact that this must be an essential step for any researcher working on MNC isolation for cell transplant or research to yield better and efficient result.

It was known for several years that infusion of Mononuclear cell from bone marrow was the promising source of stem cell therapy (Serban *et al*., 2008; kondo *et al*., 2003; kumar *et al.,* 2009; Gearge *et al.,* 2005; Wilson *et al.,* 2006; Jansen *et al.,* 2007). However, an efficient alternative approach needs to be elucidated and practiced as MNC were highly heterogeneous and possess granulocyte interface (Leblond *et al*., 2009; Scherschel and Soonpaa, 2008; Murry and Soonpaa, 2004). In spite of several investigations carried out in the field of FACS based purification methods (Broers *et al.*,2003 pruszak *et al.,* 2007; Herzenberg *et al.,* 2002), Cytotherapeutic regenerative application have not been much concentrated upon. Above all, specific focus on identification of CD34+ HSC selection by FACS or MACS sorting has been the major goal of many investigators. This was because of the fact that the hematopoiesis created interest in the "in vivo" Mechanism of biology to the scientific world since many years. Considering the properties of MSC (Donald *et al*., 2007; Chamberlain *et al.,* 2007; Nasef *et al*.,2008), it is clear that isolated homogenous HSC by CD34+sorting or lineage depletion by MACS does not possess cytotherapeutic potential. This is because HSC possesses less plasticity and inefficient chemokine property to engraft at the site of injury unlike MSC.

Much work on EP cell plasticity is not widely studied and CD 105+ endoglin which was said to be putative EP cell population is also not much focused in research. Likewise, presence of side population cells has been shown in many adult tissues and the SP Phenotype might be represented as a common molecular regulatory feature for a wide variety of stem cells (Guo y, *et al.,* 2003). A Lymphocyte count is usually part of a peripheral complete blood cell count and is expressed as percentage of lymphocyte total WBC counted. Although bone marrow is the ultimate source of lymphocytes, the lymphocytes that will become T-cells migrate from the bone marrow to the thymus where they mature. Monocyte was produced by the bone marrow from HSC precursors called Monoblasts. Also it was responsible for phagocytosis (ingestion) of foreign substances. The body granulocytes were part of the innate system and have somewhat nonspecific, broad-based activity. They do not respond exclusively to specific antigen, as do B-cells and T-cells. Ficoll-paque PLUS was an aqueous solution of density 1.077. Lymphocyte isolation using Ficoll-paque PLUS was based on methodology established through the extensive studies or boyum. Before processing granulocytes contamination are more, lymphocytes population is less. After processing lymphocytes population is increased and Granulocytes are avoided. In general lymphocytes are immature cells in bone marrow than other cells as they only mature in thymus, thus lymphocytes are said to be uncommitted and have more stem cells. The other reason o lymphocyte to have more stem cells is because of its own Bone marrow niche which is explained earlier.

VI. CONCLUSION

The present study revealed that homogenous mesenchymal stem cells with reference to CD34+, CD45+, HLA-DR+, CD73+, CD44+, CD13+, CD90+, CD105+ play as an important potent marker for mesenchymal stem cells. This research study had great therapeutic value because of the fact that heterogenous mononuclear cells has disadvantages, where it has stem cell populations with more granulocyte interface hindering the engraftment of stem cells at the site of injury. Thus more study on homogenous mesenchymal stem cell marker with reference to the above would unravel the existing mesenchymal stem cell problems and serve as a great therapeutic tool in the field of regenerative medicine.

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TABLE AND GRAPH:

GRAPH: 1 CELL COUNTING USING FACS, HSCS, MSC AND EP CELLS

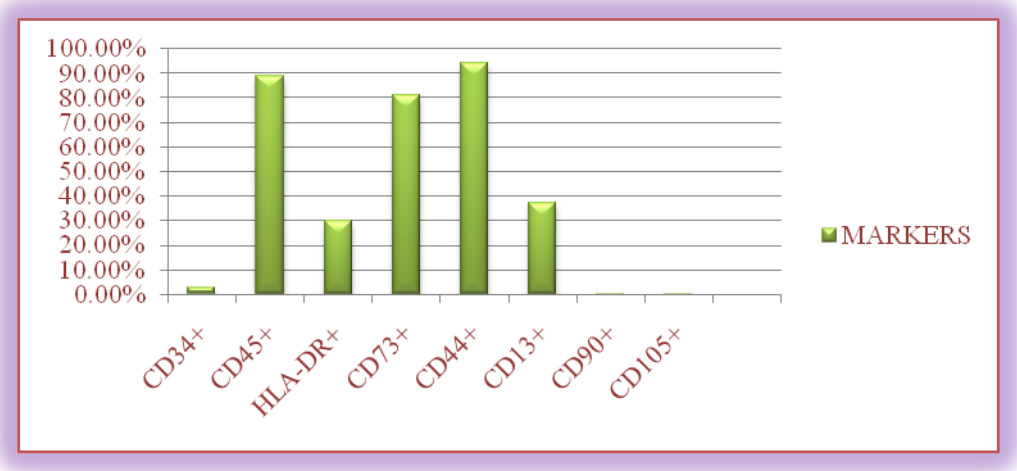

TABLE: 1 CELL COUNTING USING FACS HSCS, MSC AND EP CELLS

PARAMETERS	PERCENTAG
CD 34+	2.8
CD 45+	89.0
HLA-DR+	30.0
CD 73+	81.3
CD 44+	94.2

CD 13+	37.1
CD 90+	0.3
CD 105+	0.2

GRAPH 2: COMPARISION OF VIABLE AND DEAD CELLS

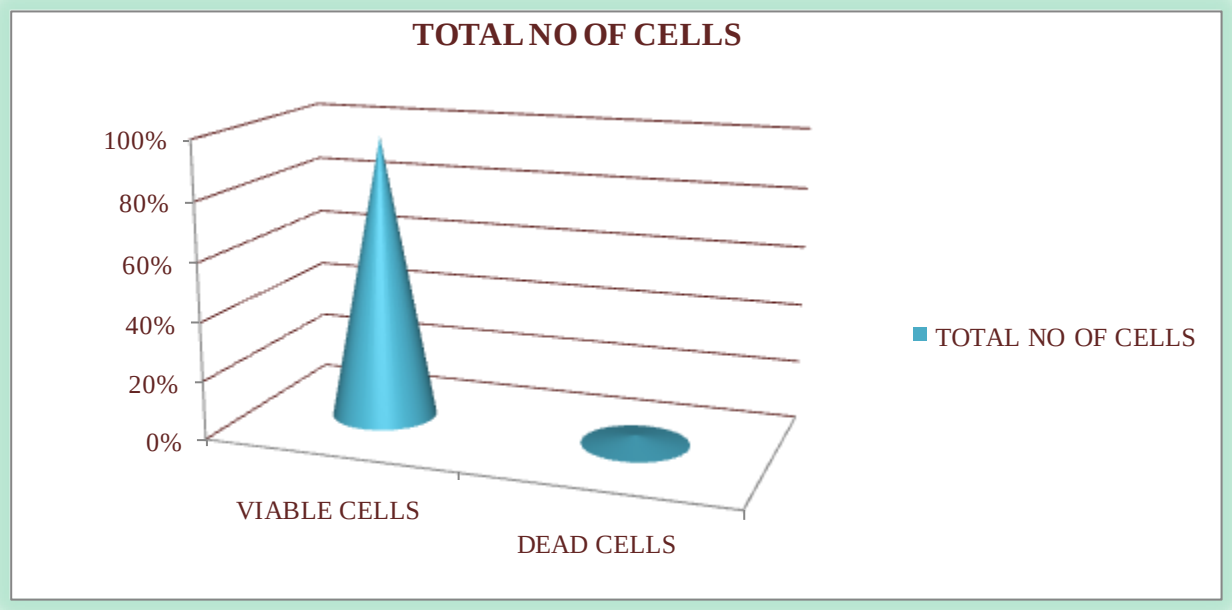

TABLE: 2 EFFICIENCY OF PROCESSING OF MONONUCLEAR CELLS

POPULATION	BEFORE PROCESSING	AFTER PROCESSING
Lymphocytes	**32.92%**	**65.20%**
Monocytes	**3.70%**	**7.30%**
Granulocytes	**40.50%**	**0.20%**

GRAPH 3: EFFICIENCY OF PROCESSING OF MONONUCLEAR CELLS