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RESEARCH ARTICLE

In vitro Expansion and Differentiation of Hematopoietic Stem Cells into Platelets.

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Abstract

The aim: This study aimed to collect mobilized HSCs by aphaeresis and culturing the cells under effect of various growth factors cocktails to generate mature megakaryocytes and platelets.

Material and Methods: The present study was conducted on 10 subjects divided into: (group 1) 5 patients of liver disease with thrombocytopenia and (group 2) 5 healthy control. The culture protocol lasted for 14 days, the process of megakaryopoiesis was characterized by flow cytometry using fluorochrome-conjugated antibodies against CD61, CD34 and CD42. Mature MKs were observed as larger cells in culture using a phase contrast inverted microscope.

Results: HSCs cultured for 14 days in growth factors cocktail revealed weak expression of CD34 and positive expression of both CD61 and CD42 for both CLD and control groups compared to those before culturing. Moreover, inverted microscope study on the 14th day revealed the presence of mature MK which can be observed as larger cells in the culture.

Conclusion: The present study revealed successful generation of MK and platelets having CD61+ve and CD42+ve surface markers from differentiation of HSCs derived from peripheral blood which is an easy accessible source with no ethical problems. This study also demonstrated that CLD doesn't affect HSCs differentiation into MK and platelets. These results together with those of previous studies constitute important implications for the development of regenerative medicine in patients suffering from thrombocytopenia.

Introduction:-

Thrombocytopenia is a common and challenging clinical disorder in patients with chronic liver disease. It is predominantly a result of portal hypertension and platelet sequestration in the enlarged spleen, but other mechanisms may contribute. The liver is the site of thrombopoietin (TPO) synthesis, a hormone that leads to proliferation and differentiation of megakaryocytes and platelet formation. Reduced TPO production further reduces measurable serum platelet counts. New therapeutic options are needed to safely increase platelet counts particularly prior to invasive medical procedures as well as to counteract therapies that further exacerbate low platelets, such as interferon (Poordad, 2007).

Platelet transfusions however are expensive and associated with a number of side effects including febrile or allergic transfusion reactions, transmission of bacterial and viral infections, circulatory congestion, transfusion-related acute lung injury and alloimmunization (Schiffer et al., 2001). The transfusion of autologous platelets is the best option for avoiding alloimmunization and infection (Pedrazzoli et al., 2000).

Stem cells are defined functionally as regeneration units responsible for the development and regeneration of tissues and organs, being capable of both self-renewal and multilineages differentiation (Weissman et al., 2001). Adult stem cells are present in a variety of organs including bone marrow. Their role is to replenish multiple mature differentiated cell types and thereby achieve long-term tissue reconstitution (Robey**,** 2000). Hematopoietic stem cells (HSCs), as an example of adult stem cells, are multipotent self-renewing stem cells that can differentiate into a number of cells in other lineages that constitute various tissues, but only those of a closely related family of cells, a property known as plasticity (Anderson et al., 2001; Schöler and Hans, 2007).

Hematopoietic stem cells are an excellent candidate for cell therapy because they are easily accessible and they can be bio-preserved with minimal loss of potency (Bryder et al., 2006). HSCs are derived from 3 sources: umbilical cord blood (UCB), adult bone marrow, and peripheral blood after treatment with recombinant human granulocyte colony-stimulating factor (G-CSF); a cytokine that mobilizes HSCs from the bone marrow and allows their harvest through peripheral blood aphaeresis (Lee et al., 2013). Transplantation of HSC from the peripheral blood results in faster hematologic recovery than HSC from bone marrow (Jansen et al., 2005).

Numerous culture protocols have been developed over the years to derive megakaryocyte (MK) and/or platelet ex vivo from human cord blood, mobilized peripheral blood and bone marrow CD34 cells as well as embryonic stem cells (Nishikii et al., 2008) most rely on the use of serum-free medium supplemented with cytokine cocktails and often consist of two or three different culture phases to improve yields (Reems et al., 2010).The aim of this study was to collect mobilized HSCs by aphaeresis and culturing cells under effect of various growth factors cocktails to generate mature megakaryocytes. This study was performed hoping to allow autologous transfusion for thrombocytopenic patients with less contamination and complication.

Material and Methods:-

The present study was conducted on 10 subjects divided into: (group 1) 5 patients of liver disease with thrombocytopenia chosen from Tropical Medicine Department, Theodor Bilharz Research Institute (TBRI) and (group 2) 5 healthy individuals with normal blood picture, negative history of liver diseases and serological markers negative for hepatitis C virus (HCV) served as a control group. Patients were recruited with ethics committee approval of Theodor Bilharz Research Institute (TBRI-IRB number 03/15) according to Helsinky Declaration. Informed consent was granted in all cases.

Routine laboratory investigations:-

- Complete blood count (CBC).
- Coagulation profile: Prothrombin time (PT), international normalized ratio (INR).
- Serological markers for HCV, HBV by ELISA.

Special investigations:-

- Estimation of HSCs percentage among peripheral blood mononuclear cells (PBMCs) assayed by Flow cytometry after labeling with CD34-PE (Phycoerythrin) labeled monoclonal antibodies following the suggested protocol (Duda et al., 2007).
- Monoclonal anti human CD61- FITC labeled and monoclonal anti human CD42a- FITC labeled were assayed by Flow cytometry.

Assay method:-

*** Mobilization:** 300µg G-CSF (neupogen) was injected once a day subcutaneously until enough stem cells are collected from the blood. This usually takes 4 days.

*** Aphaeresis:** Hematopoietic progenitor cells collected from the peripheral blood by aphaeresis. In this procedure, the leukocytes are separated from red cells and plasma following centrifugation in an extracorporeal system, usually maintained by continuous recirculation of blood through the aphaeresis device (Mikhail et al., 2012).

* **Analysis by flow cytometry**: After aphaeresis, nucleated cells were transferred into 3 sterile Wasserman tubes and resuspended up to 10^7 per 100 ul of buffer solution (PBS + 0.5% Bovine serum albumin (BSA)). The first tube is the control for the procedure with no monoclonal antibody and on which the flow cytometry is adjusted in order to obtain the basic histogram showing the main cell population and to adjust the autofluorescence region, the second and the third tube are the sample tubes in which monoclonal antibodies were added.

-10 µl of anti CD34- FITC labeled (Miltenyi Biotec.) antibody was added to both tubes.
-10 µl of anti CD42a- FITC labeled (Miltenyi Biotec.) antibody was added to the second tube.
-10 µl of anti CD34- PE labeled (Miltenyi Biotec.) antibody was added to the third tube.

The 3 tubes were incubated for 10 minutes at (2-8^{o}C) in the dark, washed and cell pellet was resuspended in a suitable amount of buffer for analysis by flow cytometry.

Culturing: The culture protocol usually lasts about 14 days. MK and platelet productions usually peak at day-14. Mature MK can be observed as larger cells in culture using a phase contrast inverted microscope. The MK culture medium consisted of:

1- Iscove's modified Dulbecco medium (IMDM).
2- Fetal bovine serum.
3- Penicillin/streptomycin.
4- Fungizone.
5- (MK culture medium): required volumes of cytokines cocktail (final concentration of 1 ng/mL stem cell factor (SCF), 30 ng/mL Recombinant human thrombopoietin (TPO), 13.5 ng/mL IL-9, and 7.5 ng/mL IL-6 (Pineault et al., 2013).

Typically the aphaeresis product contains less than 10% of the red cell content of peripheral blood on a volume basis, with measured hematocrit usually in the range of 3–4% (Mikhail et al., 2012), so after separation of mononuclear cells they were transferred to 15-mL tube complete with suitable amount of PBS and centrifuged at 228 × g for 10 min then discard the supernatant. The cells were resuspended in MK culture medium to a density of 3–6 $\times 10^5$ cells/ml after measure the cell density by hemocytometer. 10 ml of the culture medium were added to each flask. Cultures were incubated at 39 °C, 10% CO 2, 95% humidity in a humidified incubator.

At day-4, the cell cultures were mix gently, half of the cell suspension volume was removed and an equivalent volume of fresh MK culture medium added.

At day-7, the cell cultures were mixed gently and diluted with fresh MK culture medium to reach a cell density of 2 $\times 10^5$ - 3 $\times 10^5$ cells/ml, and transfer the cultures at 37°C.

At day-10 or 11, the cell cultures were mixed gently. The cell cultures were transferred to 15 or 50 ml tube(s) and centrifuged at 228 × g for 10 min and discarded the supernatant. The cells were resuspended in fresh MK culture medium to reach 3 $\times 10^5$ cells/ml.

At day-14 quantitative monitoring of the process of megakaryopoiesis cells should be characterized by flow cytometry using fluorochrome-conjugated antibodies against CD61, CD34 and CD42. Cells expressing against CD61, CD34 and CD42 emitted fluorescence signals, which was summated and multiplied in the PMTs and the computer analyzed the data as graphical scale (One Parameter, Two parameter Histograms).

In general, the sequence of differentiation of uncommitted cells toward matures MK are as follows (Pineault et al., 2013):
Hematopoietic Progenitor cells: CD34+ve CD61-ve CD42 -ve
Committed MK Progenitor: CD34+ve CD61+ve CD42 -ve
Immature MK: CD34-ve CD61+ve CD42 -ve
Mature MK: CD34-ve CD61+ve CD42 +ve

Statistical analysis:-
It was done by using software package (SPSS PC, Chicago,IL) version 18. The difference between two groups or more were analyzed for statistical significance by one way ANOVA test. All tests were considered statistically significant when P value < 0.05 and highly significant if < 0.01.

Results:-

Demographic and clinical features of the 2 studied groups are listed in Table (1):

Table (1): Demographic features of the 2 studied groups.

Item	CLD group (No 5)	Control group (No 5)
Age (years) • Range • Mean ± SD	 41-52 45.4±4.97	 30-42 36.2 ± 4.91
Sex no. (%) • Females • Males	 2 (40%) 3 (60%)	 1 (20%) 4 (80%)
Hypertension no. (%) • Present • Absent	 4 (80%) 1 (20%)	
Interferon intake no. (%) • Present • Absent	 2 (40%) 3 (60%)	

Routine laboratory data of the studied groups are shown in table (2)

Table (2): Laboratory data of the 2 studied groups.

Items	CLD group (No 5)	Control group (No 5)
Hb (g/dl) • Range • Mean ± SD	 10.20-13.8 11.90 ± 1.57	 11.90-16.20 14.04 ± 1.85
TLC (× 10^3) • Range • Mean ± SD	 21.40-46.80 34.92 ± 11.18	 22.00-51.80 31.98 ± 12.51
Platelets (× 10^3 /cmm) • Range • Mean ± SD	 81-134 109.00 ± 22.42**	 174-337 281.60 ± 77.50
ALT (I.U) • Range • Mean ± SD	 2-11 5.60 ± 3.78	 4-7 5.20 ± 1.30
AST (I.U) • Range • Mean ± SD	 2-14 6 ± 4.74	 3-8 5.00 ± 1.87

**: highly significant difference compared to the control group ($p<0.01$), Hb: Hemoglobin, TLC: Total leucocytic count, ALT: Alanine transaminase, AST: Aspartate transaminase

Flow cytometry expression before and after culturing in the 2 studied groups are shown in table (3) and figure (1and 2):

Table (3): Flow cytometry expression before and after culturing in the 2 studied groups.

Item	**CLD group (No 5)**	**Control group (No 5)**
CD34 before culturing • **Range** • **Mean± SD**	 24.5 - 42.6% 35.40 ± 6.87%	 29.5 - 47.3% 40.40 ± 7.02%
CD34 after culturing • **Range** • **Mean± SD**	 0.94 -1.89% 1.20 ± 0.44%**	 1.11-4.29% 2.6 ± 1.5%**
CD61 before culturing • **Range** • **Mean± SD**	 0 0	 0 0
CD61 after culturing • **Range** • **Mean± SD**	 35.1-73.6% 51.60 ± 15.33%**	 51.3-80.6% 69.40 ± 11.14%**
CD42 before culturing • **Range** • **Mean± SD**	 0 0	 0 0
CD42 after culturing • **Range** • **Mean± SD**	 36.3-97.9% 70.00±22.40%**	 80.9-99.6% 91.40±7.98%**

**: highly significant difference compared to before culturing ($p<0.01$)

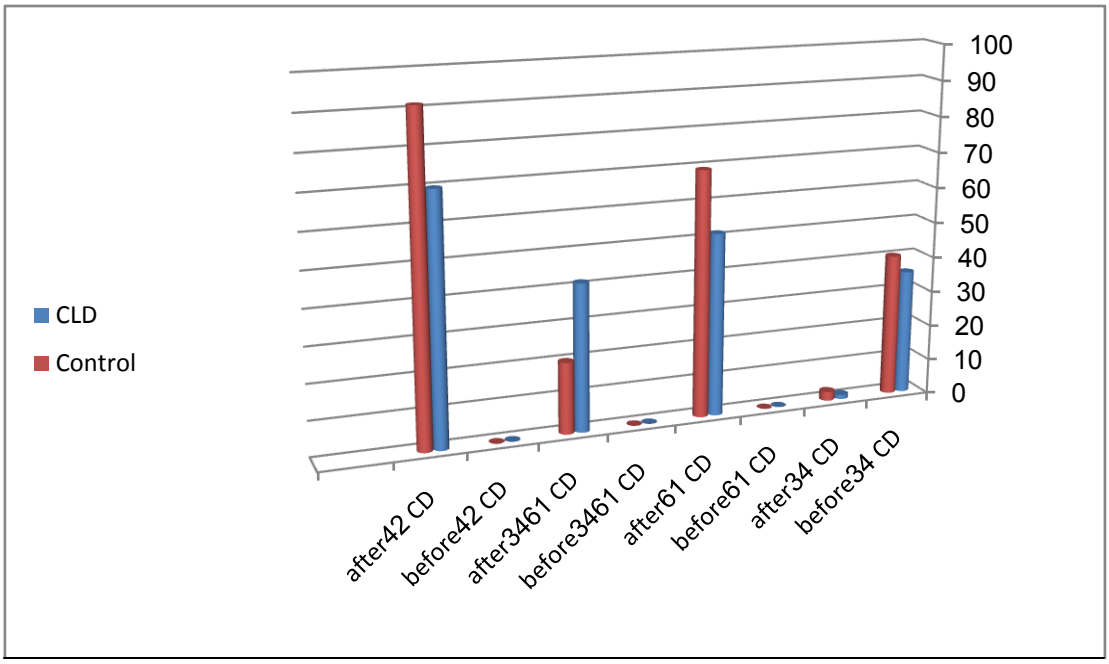

Figure (1): Flow cytometry expression before and after culturing in the 2 studied groups.

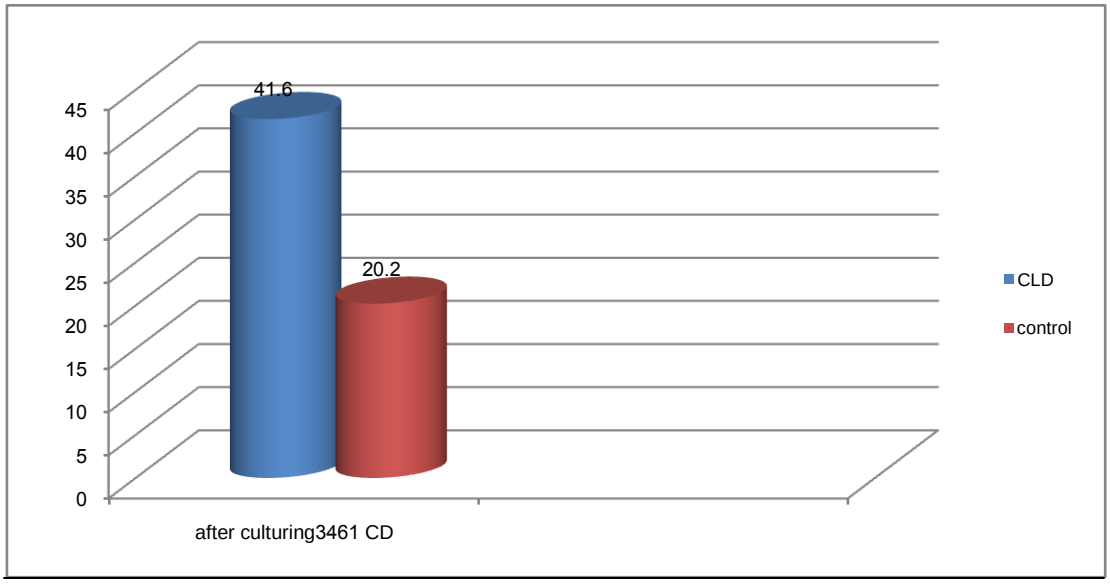

Figure (2): Expression levels of CD3461 by flow cytometry after culturing in the 2 studied groups.

Inverted microscopic appearance throughout the culturing period are shown in figures (3-9)

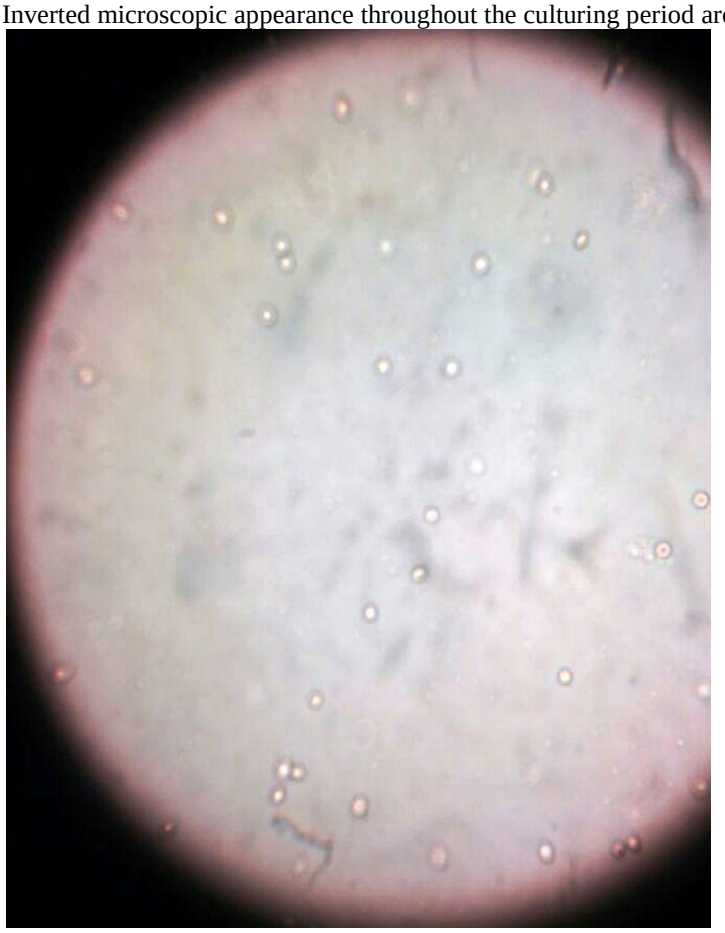

Figure (3): Day 0 culturing.

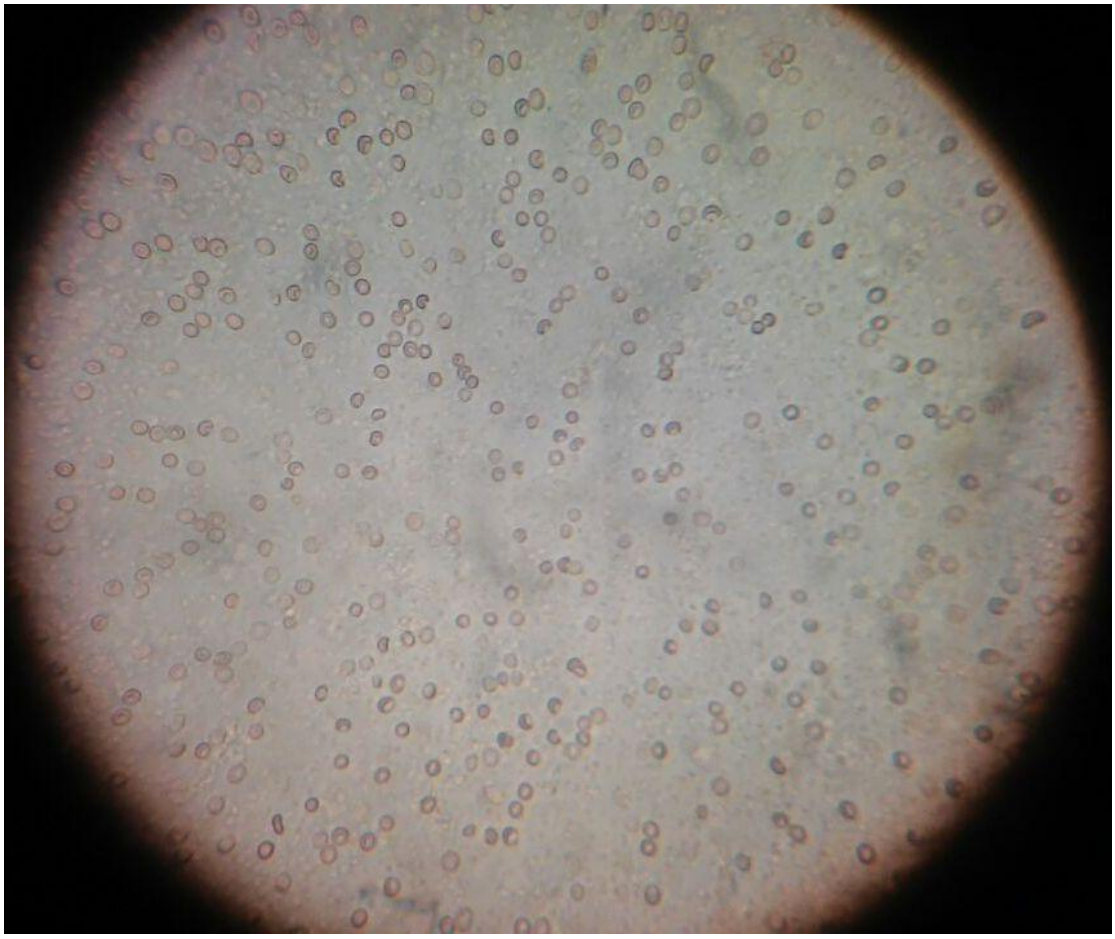

Figure (4): Day 4 culturing.

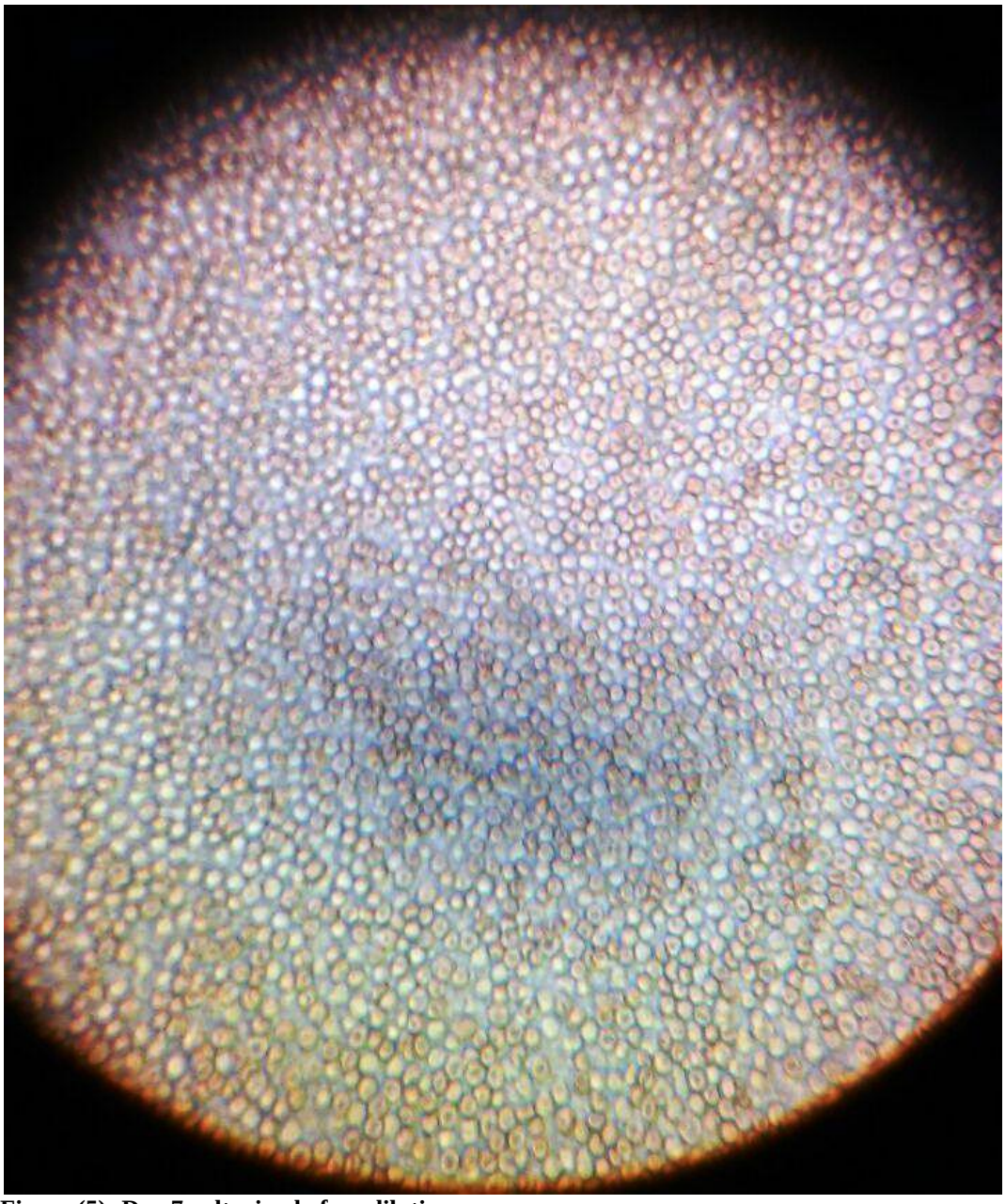

Figure (5): Day 7 culturing before dilution.

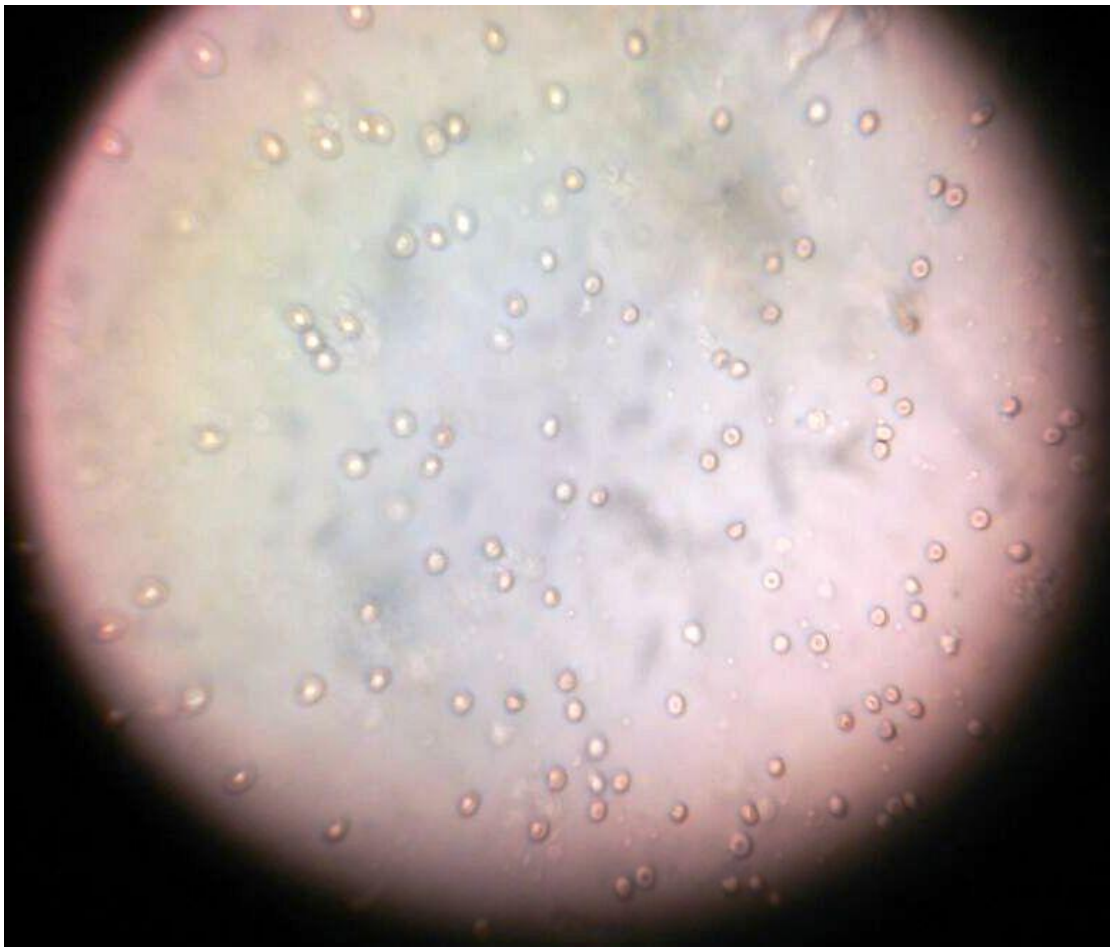

Figure (6): Day 7 culturing after dilution.

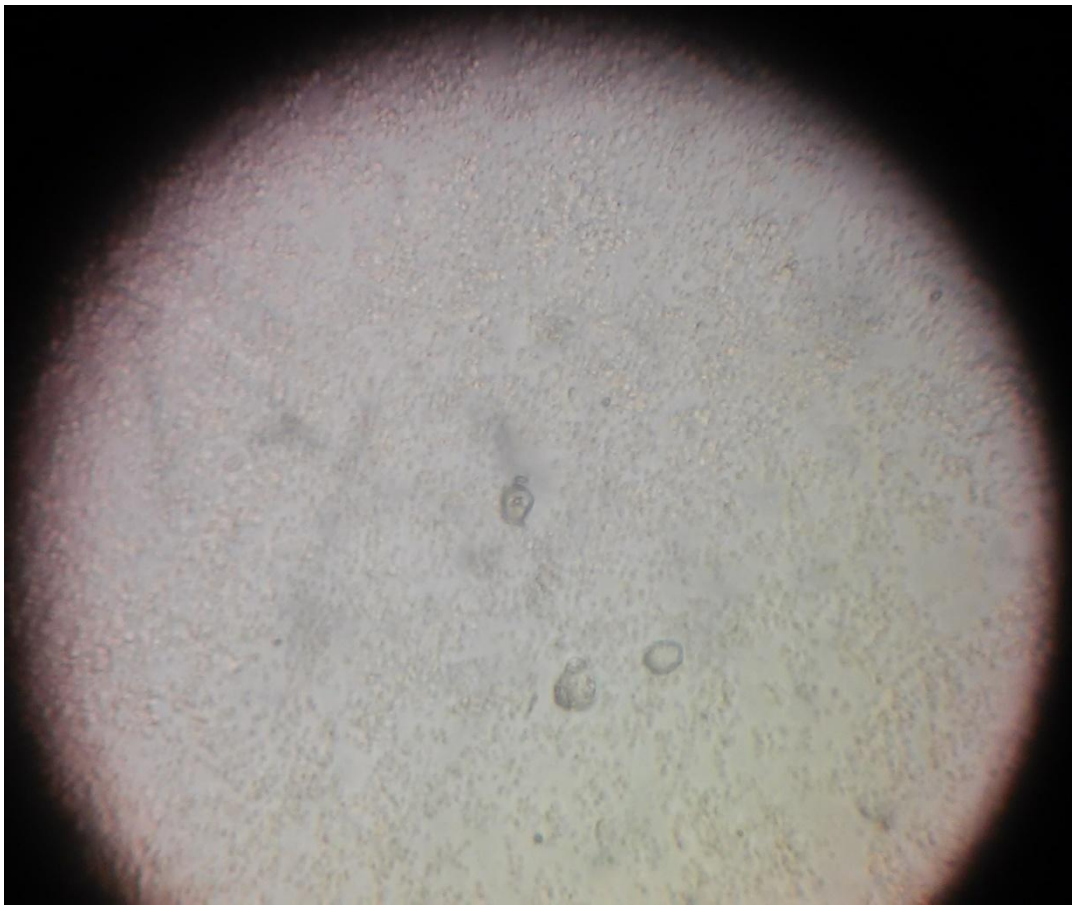

Figure (7): Day 9 culturing.

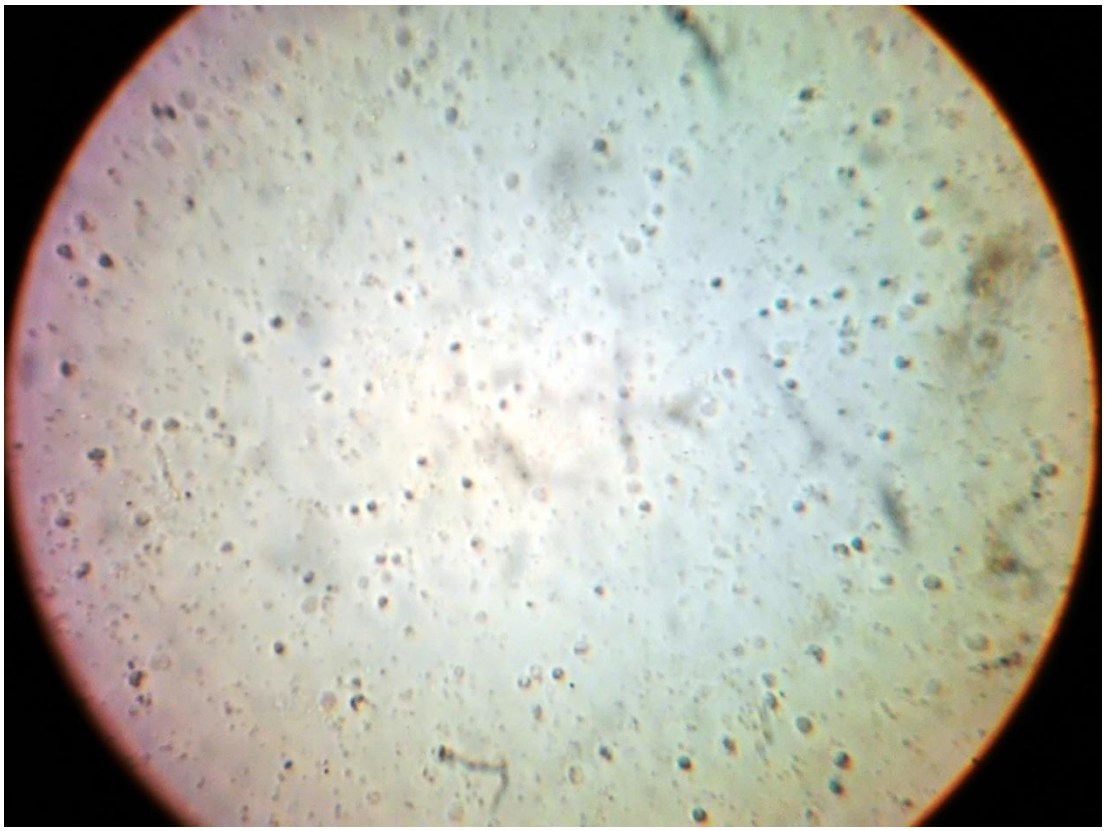

Figure (8): Day 11 culturing.

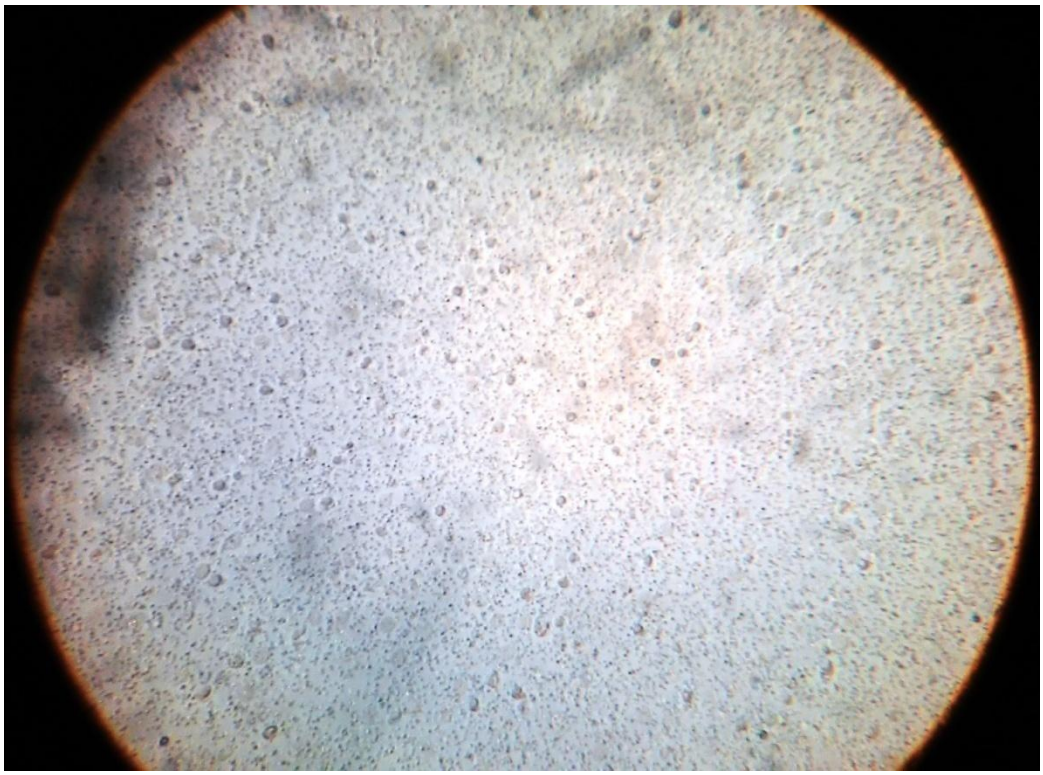

Figure (9): Day 14 culturing.

Discussion:-

Patients with thrombocytopenia are traditionally treated by platelet transfusion which exposes the patients to many risks as immune-mediated platelet destruction, allergic reaction, blood born infections like HBV, HCV and HIV, transfusion-related acute lung injury and some patients who receive a blood platelet transfusion can develop graft-vs-host disease (GVHD) (Mohanty, 2009). This study was performed hoping to allow autologous transfusion for thrombocytopenic patients with less contamination and complication.

In the present study HSCs were obtained from human peripheral blood after mobilization by granulocyte colony stimulating factor by aphaeresis. Positive expression of CD 34 marker of the un-differentiated HSCs using flowcytometric analysis was performed on the same day of collection to accurately follow the expansion and differentiation kinetics of the CD34 cells into MK and platelets. Similar was done by (Pineault et al., 2013). who obtained HSCs from cord blood.

In the present study, flowcytometric analysis revealed that culture of HSCs for 14 days in growth factors cocktail used in the trial showed weak expression of CD34 for both CLD and control groups compared to those before culturing. Moreover, cultured HSCs for 14 days demonstrated positive expression of both CD61 and CD42 in both CLD and control groups compared to those before culturing. This was in accordance with (Pineault et al., 2013) and denotes differentiation of HSCs into differentiate HSCs, MK and platelets. No significant difference could be detected between the two studied groups regarding the previous parameters.

Inverted microscope study on the 14^{th} day revealed the presence of mature MK which can be observed as larger cells in the culture. Similar picture was observed by (Pineault et al., 2013).

The present study revealed successful generation of MK and platelets having CD61+ve and CD42+ve surface markers from differentiation of HSCs derived from peripheral blood which is an easy accessible source with no ethical problems. This study also demonstrated that CLD doesn't affect HSCs differentiation into MK and platelets. These results together with those of previous studies constitute important implications for the development of regenerative medicine in patients suffering from thrombocytopenia.

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Authors' Contribution

All authors were involved in all steps of manuscript preparation.

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