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Regenerative change of Bone Marrow Hematopoietic Stem Cell (BMHSCs) on Secondary Leukemia and Lung Injury Induced as Adverse Chemotherapeutic (etoposide) Drug in Albino Rats

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Abstract

Background: Although secondary myelodysplasia leukemias was observed after some chemotherapy but the treatment of these malignancies is unclear. Thus, this study was examined clinical and pathological features to evaluate the regenerative effect of transplanted bone marrow-derived hematopoietic stem cells (BMHSCS) on the adverse effect of etoposide which induced impairment of bone marrow function, and secondary leukemia induction, in albino rats.

Methods: Two groups of albino rats were established. Group 1 was used as control group. Group 2, all rats were injected intraperitoneally with three consecutive doses of etoposide (0.15ml/ rat), then divided into (subgp A), which was sacrificed at 3th, 6th day from the beginning

of experiment and (subgp B) which was injected intravenously, with one dose of labeled BMHSCs ($0.5x10^6$ cells /rat), at the 6^{th} day from the beginning of experiment, and sacrificed at 1^{st}, 5^{th}, 10^{th} and 13^{th} day post-treatment.

Results: All rats received chemotherapeutic drug (etoposite) in subgroup A showed pancytopenia with severe leukemia, interstitial pneumonia and death. However, The Bone Marrow hematopoietic stem cell (BMHSCs) treated group (subgroup B) elucidated overall survival in all leukemic animals (induced by etoposide chemotherapeutic drug), with hypercellularity in bone marrow, and reversible in lung tissues.

Conclusions: It could be concluded that etoposide demonstrated immune suppression with myeloid leukemia, complicated with secondary lung injury. However, after the transplantation of BMHSCS, significant repairing of bone marrow and lung tissues was observed.

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Background

Cytotoxic agents are alkylating agents as cyclophosphamide, melphalan and topoisomerase inhibitors that interact with topoisomerase enzyme as etoposide, doxorubicin **[1].** Etoposide (VP-16, 4′-dimethylepipodophylloxin-9-[4, 6-O-ethylidene-beta-D-glucopyranoside]) has an anticancer drug, was firstly produced in 1966 from epipodophyllotoxin which found in the American May apple (Podophyllum peltatum) and approved for administration in 1983 **[2].** Leukemia is a disease of the blood or bone marrow, characterized by increased numbers of abnormal white blood cells **[3].** It can be divided into acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), the former is classified into eight groups (FAB 0-7) based on lineage characteristics of the blast cells, but the latter being subdivided into B- and T-cell ALL **[4].**

Materials and Methods

Experimental animals

Eighty five female Wister albino rats (each weighing 180-200 gm and two months old) were purchased from Cairo University, animal house, Egypt. The study protocol was approved by the Animal Ethics Committee at Cairo University, Egypt. All animals were maintained in a pathogen-free environment and allowed to acclimatize one week prior to the experiment in plastic cages (7 animals/ cage) inside a well-ventilated room. The animals were maintained under standard conditions (temperature of 23± 3ºC, relative humidity of 60–70%, and a 12-hour light/dark cycle), fed a diet of standard commercial pellets, and given water *ad libitum*.

Etoposide drug

Etoposide is a semisynthetic derivative of podophylotoxin used in the treatment of wide variety of neoplasms. It is white crystalline powder and sparingly soluble in methanol but, it

slightly soluble in water or ethyl ether. It manufactured in India by Fresenius Kabi Oncology Ltd and purchased from (EG Group for Medical Devices) as 5 ml vial (100mg/5ml). The drug was stored at temperature below 25°C, and protected from light and freezing. Each 1ml containing 20 mg etoposide and 30mg benzyl alcohol then dehydrated in alcohol.

Isolation and characterization of BMHSCs

Five adult female albino rats (2 months old) underwent BM harvesting by flushing the tibia and femur with Low glucose Dulbecco's modified Eagle's medium (DMEM) (Cambrex Bio Science, Walkersville, MD, USA). The mononuclear cell layer were isolated by Ficoll density media (GE Healthcare Bio-Sciences, Pittsburgh, USA) and centrifuged for 30 minute at 1800 rpm at 20°C and repeated for 10 minute, for viability using trypan blue dye exclusion test for dead cells **[14]**. Then resuspended in complete culture medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Cambrex Bio Science, Verviers, Belgium). The cells were incubated in 50cm culture flask (Falcon, Cairo, Egypt) at 37ºC in a 5% humidified CO2 incubator for 3 days. Non-adherent cells were collected and washed with fresh serum-free medium and counted by hemocytometer. BM-derived HSCs were characterized by crowded cultured cells, that are variable in size and shape and most of them appear rounded by inverted microscope and identified by staining with surface markers CD34 (surface marker for HSCs) and CD44 (surface marker for MSCs) (Beckman Coulter France S.A.S) using flow cytometric analyses which performed on a Fluorescence Activated Cell Sorter (FACS) flow cytometer (Coulter Epics Elite, Miami, USA).

Labeling of BMHSCs with iron oxide

Bone marrow derived hematopoietic stem cells (BMHSCs) were labeled by incubation with ferumoxides injectable solution (25 microgram Fe/ml, Feridex, Berlex Laboratories) in culture

medium for 24 hours with 375 nanogram/ml polylysine added 1 hour before cell incubation. Labeling was histologically assessed using Prussian blue stain (Biodiagnostic, Co, Dokki, Giza, Egypt). Feridex labeled HSCs were washed in PBS, trypsinized, washed and resuspended in 0.01 Mol/L PBS at concentration of ($0.5x10^6$ cells/ml) **[15]**.

Experimental design

Eighty adult female albino rats were divided into 2 groups (gps 1) (n=30). Group 2 (n= 50) were divided into 2 subgroup (subgp A and subgp B)

Group (1) the rats were injected intraperitoneally (i.p.) daily with phosphate buffered saline (PBS) at 1.5 ml/rat and used as a control group.

Group (2) all rats were injected (i.p.) with three consecutive doses of etoposide drug daily at (15 mg/ kg bwt) (0.15ml/ rat) dissolved in 1.5 ml (PBS) and divided into 2 subgps.

Subgp A: Ten rats were sacrificed at 3rd, 6th from the beginning of the experiment and used as etoposide induced leukemic diseases (Eto-I).

Subgp B: The remaining rats from gp 2 were injected intravenously (i.v.) in the tail vein, with one dose of labeled BMHSCs ($0.5x10^6$ cells /rat) added in 0.5ml PBS, at the 6th day from the beginning of the experiment and sacrificed at 1st, 5th, 10th and 13th day post-treatment (dpt) then used as BMHSCs treated etoposide induced leukemic disease (HSCs treated Eto-I)

The animals were examined daily along the experiment. Clinical signs, mortality and morbidity rates were recorded. Blood samples were collected at the times of sacrificed, from median eye canthus on EDTA-tubes for hematological examination and flow cytometric analysis. Blood film was done for differential leukocytic count and for differential myeloid leukemia cells by staining with Giemsa **[16]**, and others sections stained Sudan Black B **[17]** and Periodic Acid Schiff (PAS) stain **[18]** which were purchased from (Biodiagnostic, Co, Dokki,

Giza, Egypt). The rats in all groups were euthanized by xylazine (40mg/kg) and ketamine (400mg/kg) **[19]** before sacrificed**.** Bone marrow yield was collected by flushing of the femur and stained with Giemsa for cytological examination. Samples from femur bones and were collected from died and sacrificed animals in all groups for histochemical using Prussian blue stain and immunohistochemistry examinations. Other sections from femur bones and lungs tissues were collected for histopathological examination.

Flow cytometric analysis for detect myeloid leukemia

Ten µl of undiluted fluorescent labeled-CD13 and CD33 antibodies (Beckman Coulter France S.A.S), on 100 µl of blood in test tube and mix firmly for 30 seconds**,** then incubated for 15 -30 minutes at room temperature, then add 2 ml of lysing solution (PeliLyse A1, 10x diluted) and incubated for 10-15 minutes at room temperature until complete lysing, then analyzed the samples within 90 minutes **[20].** .

Histochemical examination using Prussian blue stain for detected the labelabed BMHSCs

Five microns of paraffin-embedded, unstained bone marrow sections were prepared and deparafinized then immersed in mixture of 40 ml of distilled water, 5 ml of potassium ferrocyanide and 5 ml of acid activation buffer and Stir briefly in Coplin jar 50 ml for 20 minutes then, wash well in distilled water and add 10 drops of eosin on the section then leave to act 10-15 seconds, then wash again in distilled water. After that, dehydrate in ascending alcohols, clear in xylene, then examined using light microscopy to ensure homing of Labeled BMHSCs in the bone marrow yield **[21].**

Immunohistochemistry (CD34 immunostaining) for cellularity of BMHSCs

This method used DakoCytomation's Envision system and a polyclonal primary rabbit $CD34^{+}$antibody purchased from (Biorbyt, Cambridge, England) which is marker for progenitor

hematopoietic stem cells. Five micron thick serial sections were prepared on positive charged slides. Antigen retrieval was performed using heat epitope antigen retrieval method by programmed a PT-Link (Dako, Glostrup, Denmark) containing Envision^{TM} FLEX target retrieval solution, High pH (50^{x}) (Dako, Glostrup, Denmark) for 20 min. Afterward, 100 µ from diluted primary $CD34^{+}$antibody (1:200) was applied to all slides except negative control slides (0.1ml of antibody diluents was applied without primary antibody) at 4°C for overnight, then washed 3 times (20 minute each) using Envision^{TM} FLEX FLEX Wash Buffer (Dako, Denmark) and treated with Envision^{TM} FLEX/HRP Buffer (Bioscience) for 30 minutes. Peroxidase compatible chromogen (DAB) was applied to each slide. Slides were stained with Mayer's Hematoxylin for 3-5 min and washed distilled water **[22]**.

Pathological examination

Specimens from femur bone (treated by EDTA (10%) for decalcification and lungs tissues, were collected from all groups and fixed in 10% neutral buffered formalin (NBF), dehydrated in alcohol and embedded in paraffin wax. Sections about 5µm thickness were prepared and stained with Harries hematoxylin and eosin for histopathological examinations **[23]**.

Statistical analysis

Statistical analysis was done using one-way analysis of variance (ANOVA). It was done to compare between control and other treated groups, followed by post-hoc analysis (Dunnett's test) using SPSS (Statistical Package for Social Sciences) version 17 according to **Borenstein [24]**. Data were presented in the form of mean ± Standard Deviation. The difference was considered statistically significant when $p < (0.05)$.

Results

Identification and purification of BMHSCs

BM-derived HSCs were characterized by crowded cultured cells, that are variable in size and shape and most of them appear rounded by inverted microscope**.** The identification and purification of HSCs by Flow cytometric analysis of immunophenotype based on cell surface markers showed that these cells expressed high level of CD34 and negative for CD44 cell marker indicating that BM-HSCs were isolated**.**

Hematological findings

Hematological findings, demonstrated no significant changes in most of blood constituents, (red blood cells (RBCs) count, hemoglobin concentration (Hb), packed cell volume (PCV), red cells indices and platelets count) in all rats of (subgp A, B) when compared with the control group (gp 1) or compared with each other. While, white blood cells (WBCs) showed significant decrease (leukocytopenia) in their count in the rats of subgp A and subgp B at 1st dpi, when compared to gp 1 and subgp B, at 5th, 10th, 13th dpt, but 5th, 10th dpt, displayed highly significant increase when compared to others groups **(Fig. 1)**.

In addition, differential count of white blood cells demonstrated significant decrease in neutrophil cells in subgp A and subgp B at 1st dpi in comparison with gp 1 and subgp B at 5th, 10th, 13th dpt, which was gradually increased but not reach to gp 1. Monocyte cells displayed significant decrease in of subgp A and subgp B at 1st, 5th dpt, in comparison with gp 1 and subgp B at 10th and 13th dpt, which reached to normal. Lymphocyte cells were significantly decreased in subgp A when compared to gp 1 and subgp B, which were significant increase but still unreached to normal group (gp 1). Leukemic cells indicated by significantly increased in hypersegmented neutrophil cells and myeloblastic leukemia cells in subgp A and subgp B at 1st dpt when compared to gp1. These cells displayed gradually significantly decreased in the

percentage in subgp B at 5th, 10th, 13th dpt, when compared to subgp A but not reach to normal in gp 1 **(Fig. 2)**.

Blood Film

By stained with Giemsa stain for detection of leukemic cells

Blood films exhibited normal mature erythrocytes and leukocytes in control group (gp 1) **(Fig. 3a)**. Meanwhile, all rats in subgp A at 3rd, 6th dpi and subgp B at 1th dpt displayed myeloblastic leukemia cells, hypersegmented neutrophil cells **(Figs. 3b)**. However, the rats in subgp B at 5th dpt showed dysplastic neutrophils with ringed nucleus **(Fig. 3c)** and myeloblast leukemic cells. Moreover, subgp B at 10th, 13th dpt showed disappeared in blast cells with significantly hypersegmented neutrophil cells and dysplastic neutrophils with ringed nucleus in some rats**.** Other rats in the same group particularly, at 13th dpt, showed apparently normal lymphocytes and monocytes **(Fig. 3d)**.

By stained with Sudan Black B (SBB) stain for detected myeloblastic leukemia

Blood films showed negative SBB stained mature white blood cells in control group **(Fig. 4a)**, while, all rats in subgp A showed positive myeloblastic leukemia cells (black cells) and negative stained lymphocyte cell when compared to gp 1 **(Fig. 4b)**. However, the rats in subgp B in all sacrificed noticed decreased number of positive SBB stained myeloblastic leukemia cells with appearance of negative stained mature WBCs in comparison with subgp A **(Figs. 4c, d)**.

By stained with Periodic Acid Schiff (PAS) stain for detected lymphoblastic leukemia

Blood films showed negative PAS stained mature white blood cells of the control group **(Fig. 5a)**, besides subgp A appeared negative PAS stained myeloblastic leukemia cells **(Fig. 5b)**.

Flow cytometric analysis

Flow cytometric analysis of peripheral blood of rats exhibited no expression of cell surface myeloid leukemia markers CD13 and CD33 in control group (**Fig. 6a)**. However, all rats in subgp A and subgp B at 1st dpt displayed higher expression of their myeloid leukemia markers curves when compared to control group **(Fig. 6b)**. Meanwhile, this curve elucidated lower expression in subgp B at 5th dpt **(Fig. 6c)**, and continued to decreased at 10th, 13thdpt when compared to subgp A **(Fig. 6d)**.

Bone marrow smear stained with Giemsa stain for detection cellularity of bone marrow

Bone marrow smear showed normal appearance, with different developmental stages of stem cell lines in all rats in control group **(Fig. 7a)**. However, all rats in subgp A particularly at 6th dpi noticed severe hypocellularity (loss of bone marrow cells) **(Fig. 7b)**. Increase of cellularity with different developmental stage of stem cells in bone marrow, with dysplastic leukemic cells and hypersegmented neutrophilic cells was appeared in subgp B particularly at 5th dpt **(Fig. 7c),** hypercellularity observed in bone marrow reached to normal appearance mainly at 13th dpt, different than those observed in subgp A **(Fig. 7d)**.

Histochemical analysis (Prussian blue stain) for detection of BMHSCs homing in bone marrow:

Bone marrow yield showed negative staining with Prussian blue of control rats and subgp A **(Figs. 8a, b)**. Meanwhile, bone marrow yield of the rats of subgp B at 1st dpt displayed multiple positive stain spindle and cuboidal stem cells **(Fig. 8c)**, decreased at 5th dpt, and disappeared at 10th, 13th dpt **(Fig. 8d)**.

Immunohistochemistry (CD34 immunostained progenitor BMHSCs)

Bone marrow progenitor stem cells (BMHSCs) analysis

The rats in subgp A displayed significant decrease in CD34 immunostained bone marrow progenitor stem cells (BMHSCs), as well as in subgp B at 1st dpt, when compared to control group. While, the animals in subgp B at 5th, 10th dpt were significantly increased in CD34 immunostained bone marrow progenitor stem cells compared to subgp A and subgp B at 13th dpt which was significantly decreased in CD34 immunostained bone marrow progenitor stem cells compared to control group **(Fig. 9).**

Bone marrow progenitor stem cells (BMHSCs) microscopical findings

Positive of round cells CD34 immunostained bone marrow progenitor stem cells were detected in control group **(Fig. 10a)**. While, few round cells positive immunostaining cells with CD 34 was observed in the bone marrow of the rats in subgp A mainly at 6th dpi and subpg B at 13th dpt **(Figs. 10b, d)** but increased the positive spindle cells of immunostaining CD34 in subgp B at 1st ,5th, 10th dpt **(Fig. 10c)**.

Pathological findings

Depression, emaciation with loss of appetite was detected in the etoposide group particularly at 6th dpt, besides mortality rate reached to 50% and morbidity was 100%. In comparison, the ratio of mortality in subgp B at 1st, 5th dpt reached to 12% and morbidity was 70%, and disappeared after 6th day from treatment with HSCs. Severe to moderate congestion in lungs particularly in subgp A. Histopathology of bone marrow in all mortalities and sacrificed animals in subgp A which infected with (Eto-I) at 3rd, 6th dpi and in subgp B which treated with (BMHSCs) at 1st dpt, showed pancytopenia (myelosuppression) characterized by increasing of fat cells and RBCs, replaced the necrotic hematopoietic cells, with presence of myeloid leukemia (blast) cell infiltrated the peripheral part of bone marrow, and severe dysplastic leukemia cells (neutrophils with rounded nucleus) encountered the central part of bone marrow in compared to

control group which appeared normal structure **(Figs. 11a, b, c)**. Meanwhile, subgp B, particularly 5th dpt displayed increased production of hematopoietic stem cells with decreased of fat cells and RBCs. However, myeloid dysplastic leukemia cells still represented with no myeloid blast in bone marrow in most rats **(Fig. 11d).** After that, hypercellularity manifested complete production of hematopoietic cells with decrease in fat cells and RBCs and reduction in myeloid dysplastic leukemic cells in subgp B particularly at 10thdpt **(Fig. 11e).** Bone marrow in subgp B at 13th dpt appeared apparently normal in architecture with complete disappeared of myeloid blast cells **(Fig. 11f)** but few myeloid dysplastic leukemic cells still noticed

In the same time, the lungs with the rats treated with (Etop-I) (subgp A), showed hyperplasia and desquamation in the epithelial lining of the bronchioles with neutrophilic cell debris led to obliteration of their lumen **(Fig. 12a),** besides edema in interstitial tissues. Alveolitis characterized by swollen in pneumocytic cells with aggregation of inflammatory cells mainly macrophages, neutrophils with few eosinophils. Thrombosis was observed with thickening in the wall of peribronchial blood vessels. Meanwhile, interstitial pneumonia manifested by aggregations of leukocytic cells and fibrous tissues proliferation, besides emphysematous areas showed in subgp A particularly at 3th, 6th dpi **(Fig. 12b)**. Bronchiolitis characterized by metaplasia in the epithelial lining in bronchioles with inflammatory edema leading to thickening in their wall **(Fig 12c)**, in addition to hyperplasia in peribronchial lymphoid tissues in subgp B at 1st, 5th dpt **(Fig. 12d)**. However, subgp B at 10th dpt noticed focal areas of hyperplasia in the septal cells with round cells infiltrated the alveolar tissue with collagen fiber **(Fig. 12e)**, progressed to mild interstitial inflammation with increase of collagen fiber, leading to regeneration with complete healing in lungs tissues in subgp B at 13th dpt **(Fig. 12f).**

Discussion

Acute myeloid leukemia (AML) means that infiltrated bone marrow, blood, and other tissues with malignant cells of the myeloid lineage **[25]**. Our work was carried out on group of albino rats injected (i.p.) with three consecutive doses of etoposide drug at (15 mg/ kg. bwt) and sacrificed at 3rd, 6th days from the beginning of experiment (subgp A), some of them (subgp B) followed by one dose of labeled BMHSCs ($0.5x10^5$ ml/rat) (i.v.) at 6th dpi, then sacrificed at 1st, 5th, 10th and 13th days post-transplantation of BMHSCs. Cell surface myeloid leukemia antibodies CD13 and CD33 were used, confirmed myeloid leukemia using Flow cytometric analysis in etoposide group **[26].** Activated natural killer cells (NK) cells and T cells express CD33 **[27],** on the majority of AML cells and the level of CD33 seems to correlate with the disease prognosis **[28].** Histochemical analysis (Prussian blue stain) and immunohistochemistry using CD34 (surface marker for primitive BMHSCs) were used, conformed homing of BMHSCs in the treated group.

Although, etoposide is a very important anticancer agent and the primary cytotoxic target for it is topoisomerase II inhibitor. Our study revealed positive myeloid leukemia (myeloblastic leukemia cells, dysplastic neutrophil cells (ring cells) and hypersegmentated neutrophils) after three consecutive doses of etoposide (subgp A). Our results may attributed to that secondary AML may be induced dependent on treatment schedule and cumulative dose of etoposide, particularly after multiple intravenous doses **[29, 30, 5].** Other authors, **Bokemeyer [31]** elucidated that high-dose etoposide, especially higher than 2.0 g/m^2 of cumulative dose, increased risk for developing secondary leukemia. The leukemic cells in our work were confirmed by giemsa, Sudan black b, while it negative by periodic acid Schiff. Simultaneously, myelosuppression with leukopenia in blood film were detected in etoposide group (subgp A). Same as lesions as, loss of cellularity of hematopoietic stem cells with presence of aggregated

myeloid of dysplastic neutrophils (ringed nucleus**)** leukemia cells, were observed histologically in bone marrow tissue. **Lovett [32]** who reported that etoposide enhanced DNA-topoisomerase II cleavage complexes within DNA substrates that contained the mixed lineage leukemia *(MLL)*. Upon that, **Stanulla [33]** said, continuous exposure to etoposide can detect DNA-topoisomerase II cleavage complexes in hematopoietic progenitor cell line. However, different studies discovered that, secondary leukemias induced by etoposide, was associated with translocations of (*MLL*) gene at human chromosomal band 11q23 **[34].** This *MLL* gene spans 100 kb, has important functions in embryogenesis and hematopoiesis **[35, 36]** *MLL* fusion proteins induce high expression of *MEIS1* and *HOX* genes that inhibit differentiation and induce stabilization of immature hematopoietic cells **[37].** Some studies assessed the rearrangements of the *MLL/ALL1* gene with 20% of acute lymphoblastic leukemias (ALL) and with 5–6% of acute myeloid leukemias (AML) **[38, 39].** In other hand, **Ezoe [5]** found that etoposide has a promote effect on cleavage and illegitimate repair along all chromosomes, but the specific oncogenic fusions with *MLL* lead to a tumorigenesis, in a hematopoietic stem-cell subpopulation. Due to changes found in bone marrow by repeated doses of etoposide in rats in (subgp A), reflect the presence of lung asthma which manifested by obstruction in bronchioles with interstitial pneumonia. This attributed to that chemotherapy is a toxic substance that reduces humeral and cell- mediated immune response**,** leading to lung asthma **[40]**. **Kim [41]** who reported that drug-induced anaphylaxis is a main deterioration in patients receiving chemotherapy, as etoposide, manifested by cough, chest discomfort, dyspnea, and sweating.

From previous reports, there are strong association established between the hematologic malignancies risk and severe asthma in lungs, attributed to allergy-induced by cytotoxic agents and immune suppression. The above mention results (in etoposide group) in bone marrow tissues

were disappeared gradually after transplanted with BMHSCs (subgp B) and less detectable particularly at 13th dpi. As well as lung damaged were progressed to normal after remodeling of bone marrow yield. The simultaneously remodeling injury in bone marrow and lung tissues after the transplantation with BMHSCs (Subgp B) are correlated with activity of immune system, which has elicited cellular and humoral mechanisms that develop natural immune responses against tumors **[42].** Current work, studied the significant of HSCs on myloid leukemia and allergic lungs. HSCs give a series of progenitors that produce cells of a given type such as multipotent progenitors (MPPs) that give common myeloid progenitors (CMPs) which result either megakaryocyte and erythrocyte progenitors (MEPs) or granulocyte and macrophage progenitors (GMPs) **[43].** Meanwhile, early lymphoid progenitors (ELPs) able to producing T, B, and NK cells which count prolymphocytes or common lymphoid progenitors (CLPs) then become pre-B cells **[44- 46].** Also, HSCs can generate multiple extracellular growth factors cytokines as (GM-CSF) which both regulate proliferation and differentiation of the precursor stem cells **[47, 48]** and predominantly appeared on the granulocyte/macrophage lineages, resulting in more production of mature neutrophils and macrophages **[49].** Additionally to GM-CSF, there are cytokines and extracellular matrix molecules are responsible for reverse cytopenia and mobilize the immune system against cancer disease **[50]**. Moreover, allogeneic progenitor cell transplantation contained more T lymphocytes and natural killer cells **[51].** T cells inhibit the production of leukemic colony-forming unit granulocyte-macrophage (CFU-GM) colonies because of higher expression levels of the protein in tumor cells as Proteinase-3 **[52]**. In addition to, **Aswald [53]** discovered other component from the innate immune system (autologous Gamma-Delta T (GD-T) cells) have a role as potential anti-cancer immune effector cells, they found that this cells appeared fewer in peripheral blood in acute myeloid leukemia (AML) (prior

to chemotherapy) but significantly increased after induction therapy. Nevertheless, eosinophils act as central effector cells in allergic inflammation, in addition to mast cells and basophils **[54]**, but recently discovered the antitumor activity for their where, eosinophils express both FcεRs, should be able to interact with tumor-targeted IgE to enhance these anti-tumor effects **[55].** The IgE class has antibodies found in blood and extracellular fluid, involved type I hypersensitivity (anaphylactic/allergic) reactions, and there have cancer therapy properties **[56]**. Eosinophils appeared scattered malignant tissues, known as tumor-associated tissue eosinophilia (TATE), which has been associated with good prognosis in carcinoma cell **[57].** Basophils develop from $CD34^{+}$ progenitors in the bone marrow, involved in allergic/anaphylactic (type I hypersensitivity) reactions, and express FcεRI, secrete T_H2 (T helper 2) cytokines, and release histamine, lipid mediators, chemokines, and cytokines after activation in circulation same as eosinophils**,** besides mast cells and macrophages, which express both FcγRI and FcεRI, can sensitized with anti-tumour-specific IgG or IgE **[58,59].** Lung in asthma usually showed blood vessel proliferation in the mucosa and submucosa lead to increased blood flow and microvascular permeability, and edema formation in alveolar and bronchiolar walls **[60].** Numerous factors of cytokines of the CXC family expressed from basophils to regulate the inflammatory reaction for angiogenesis, tissue repair and new tissue generation **[61].**

Conclusion

It could be concluded that sequential doses of etoposide consider a highly toxic on the blood and tissues in rats where it exhibit immune suppression resulting anaplastic changes in progenitor bone marrow cells particularly acute myeloid leukemia and the development of pulmonary disease during or following chemotherapy due to anaphylaxis. Meanwhile, after

transplantation of BMHSCS, hypercellularity with mild leukemic cells in bone marrow was observed and repairing in lung tissues.

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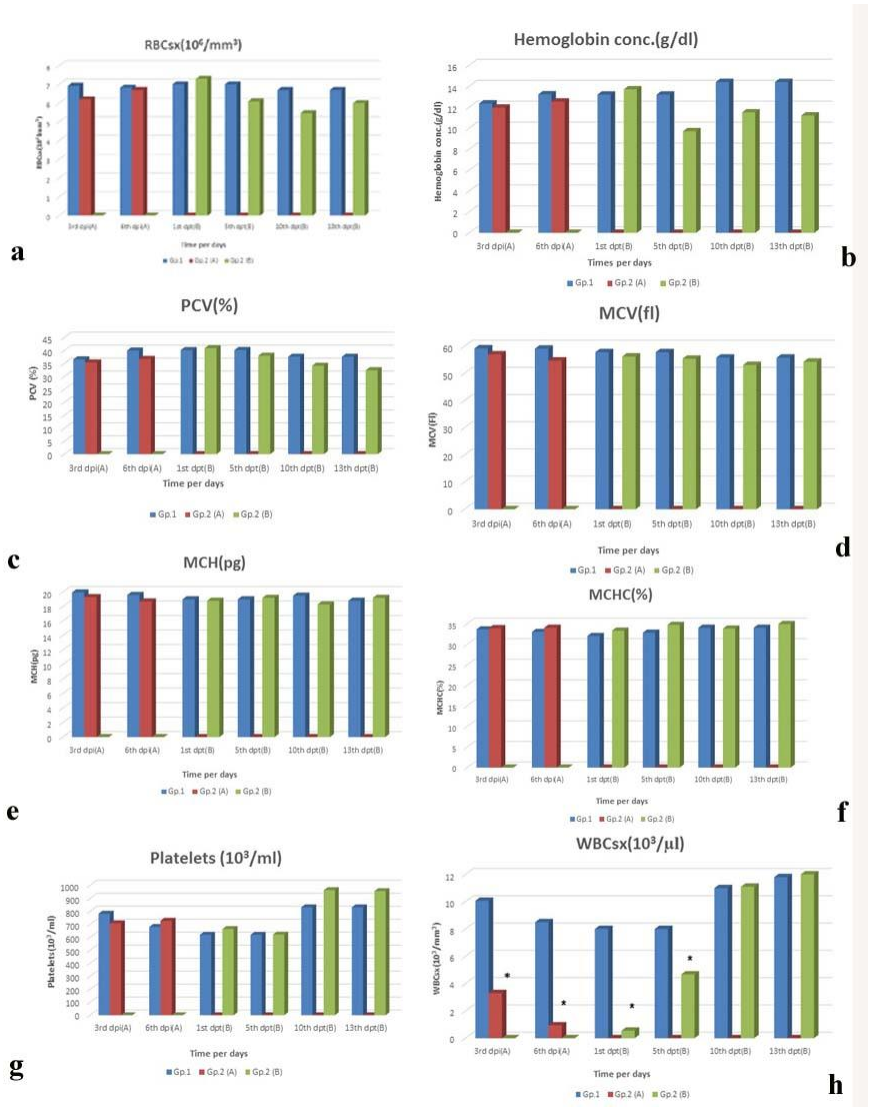

Figure 1: Mean of the red blood cells count (RBCs x10^6/mm3), hemoglobin concentration (g/dl) ,packed cell volume (PCV %), corpuscular volume (MCV) (fl), mean corpuscular hemoglobin (MCH) (pg) , mean corpuscular hemoglobin concentration (MCHC) (%) , platelet count (x10^3/ml) and white blood cells count (WBCs x103/mm3) in (control group), (subgp A, B).

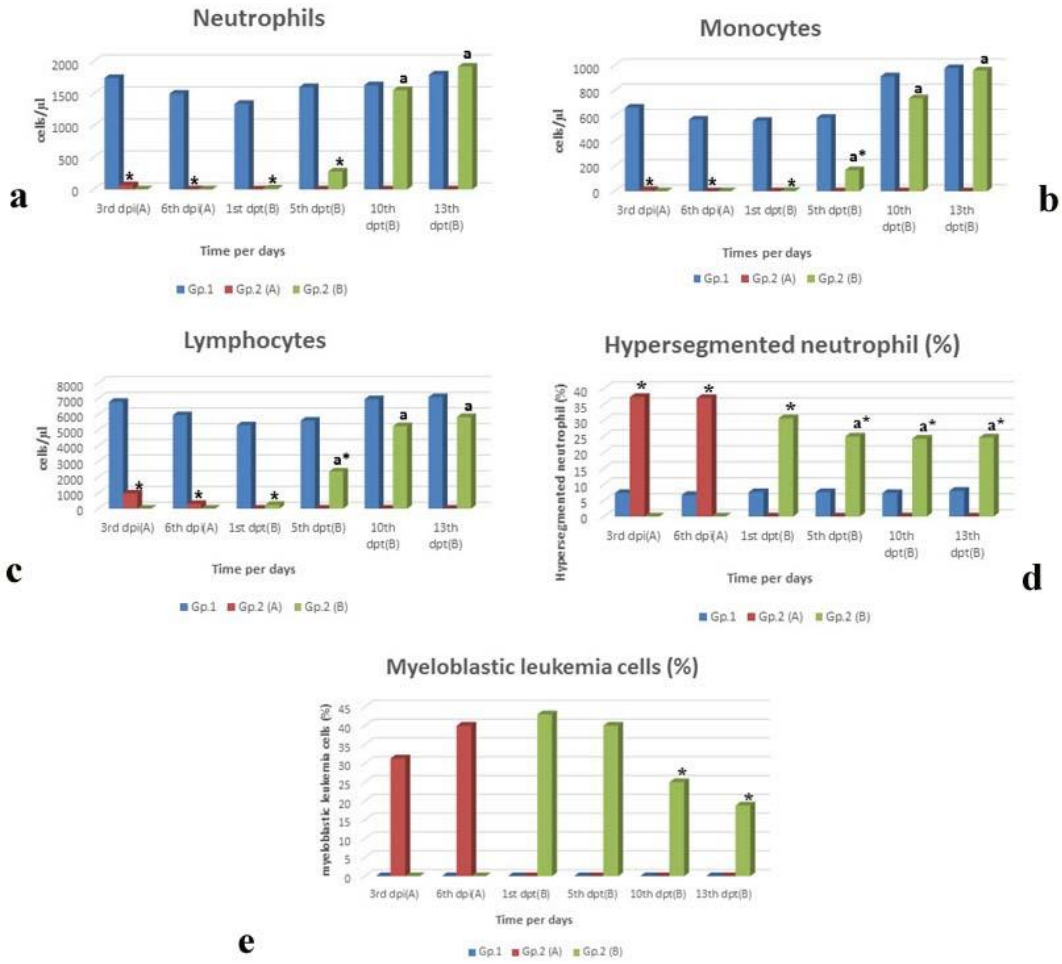

Figure 2: Mean of the Neutrophil (%), Monocyte (%), Lymphocyte (%) , Hypersegmented neutrophil (%) and myeloblastic leukemia cells (%) of rats in (control group), (subgp A, B).

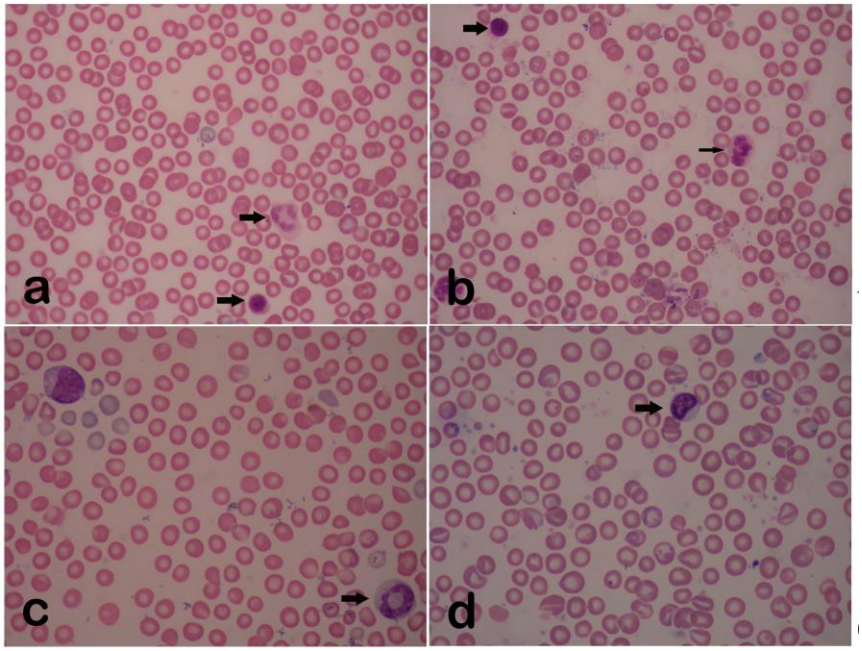

Figure 3: Blood film stained with Giemsa, (a) normal mature neutrophilic cell (thick arrow) and lymphocytic cell (thin arrow) in control group, **(b)** myeloblastic leukemia cells (thick arrow), hypersegmented neutrophilic cell (thin arrows) and **(c)** dysplastic neutrophil**,** with ringed nucleus in (subgp A, B), at 1st, 5th dpt**, (d)** normal monocyte cell at 13th dpt (arrow). **(Giemsa stain., x 100)**

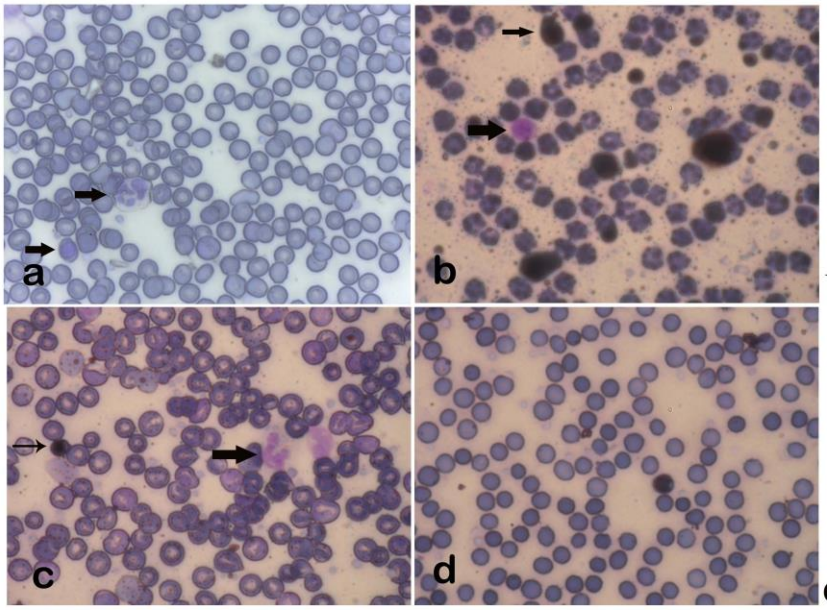

Figure 4: Blood film stained Sudan black b, (a) negative SBB stained mature white blood cells (arrows) in control group**, (b)** severe positive SBB stained myeloblastic leukemia cells (thin arrows) and negative stained lymphocyte cell (thick arrow) in **(**subgp A, B) at 1st, 5th dpt, **(c, d)** few number of positive SBB stained myeloblastic leukemia cells (thin arrow) with negative stained of mature leukocytes (thick arrows) at 10th, 13 th dpt. **(Sudan black b stain, x 100)**

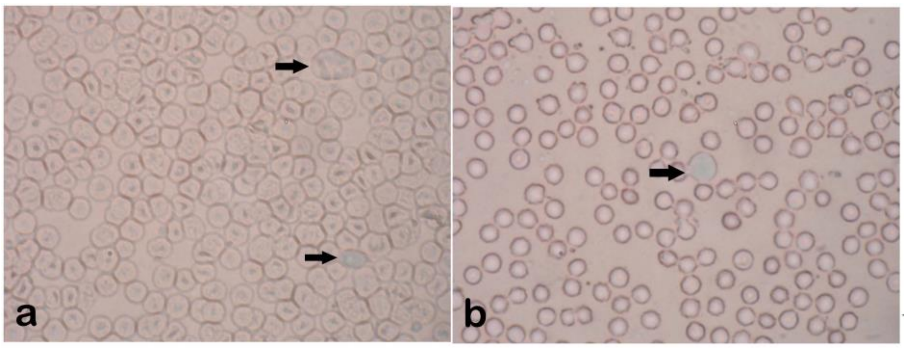

Figure 5: Blood film stained by periodic acid Schiff (PAS), (a) negative PAS stained mature white blood cells (arrows) in control group, **(b)** negative PAS staining myeloblastic leukemia cell in (subgp A) (arrow). **(PAS stain, x1000)**

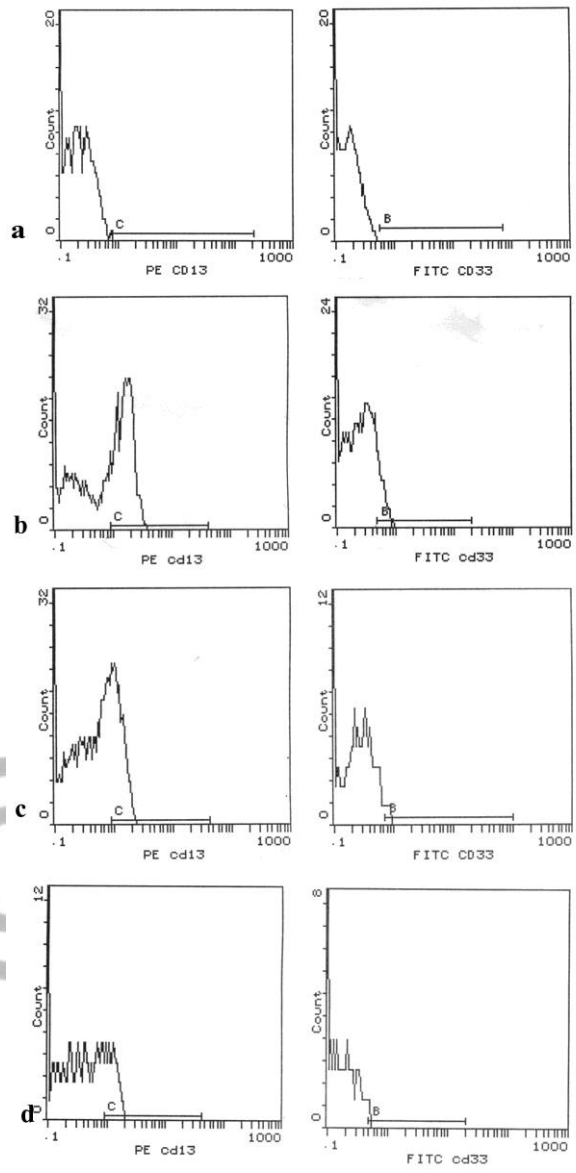

Figure 6: Histogram of peripheral blood, (a) negative of myeloid markers CD 13 and CD 33 in control group**, (b)** higher expression of myeloid markers CD13 and CD33 curve in (subgp A)**, (c)** lower expression of myeloid markers in (subgp B) at 5th dpt , **(d)** continuous decreased in expression at 10th, 13th dpt**.**

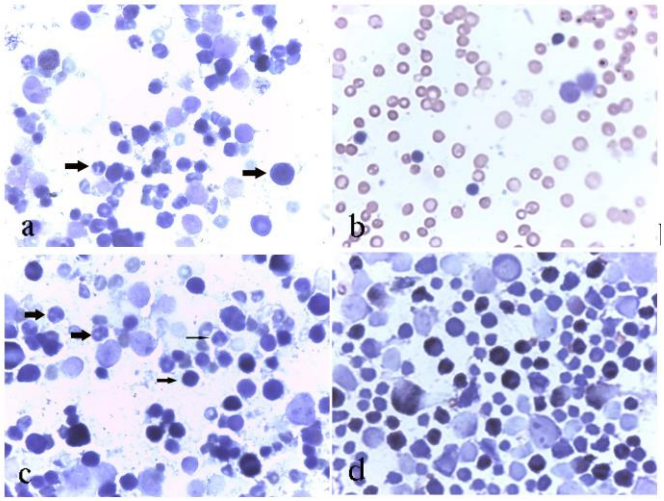

Figure 7: Bone marrow smear, **(a)** difference of developmental stages of stem cells lines in control group**, (b)** severe lost of bone marrow cells in subgp A at 6th dpi, **(c)** dysplastic cells with hypersegmented neutrophils and different developmental stage of stem cells **(arrows)** in subgp B at 5th dpt, **(d)** increasing cellularity of bone marrow in subgp B at 13th dpt. **(Giemsa stain, x1000)**

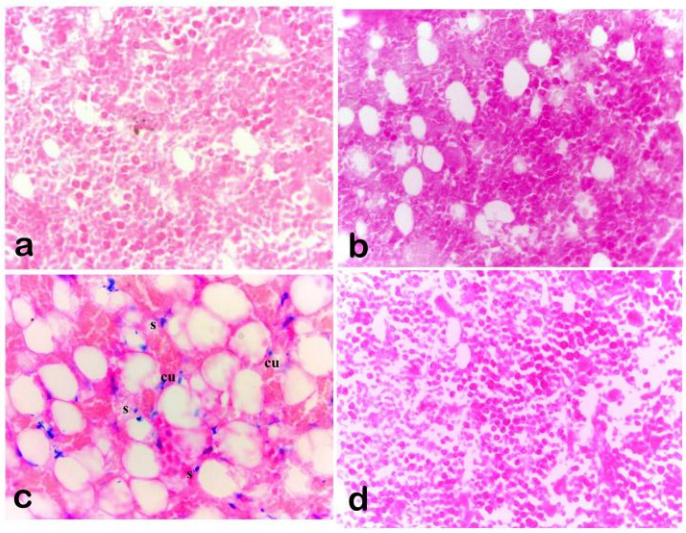

Figure 8: Bone marrow yield, **(a, b)** negative staining with Prussian blue in control group and (subgp A), **(c)** positive Prussian blue stain for multiple spindle and cuboidal cells (s) in subgp B at 1st dpt, **(d)** no positive Prussian blue cells in subgp B at 13th dpt. **(Prussian blue stain)**

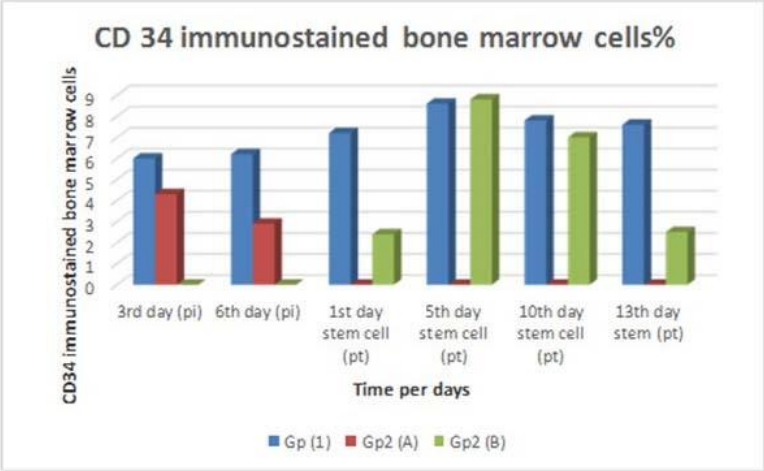

Figure 9: Mean of the CD34 immunostained cells % of rats in (control group), and (subgp A, B)

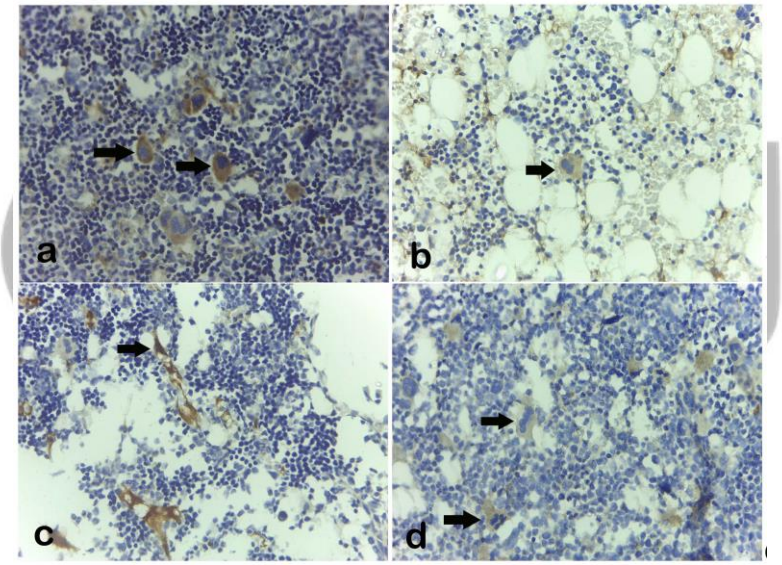

Figure 10: Bone marrow stained with CD 34 immunostaining, (a) several positive round cells stained with CD 34 (arrows) in control group, **(b, d)** few positive round cells stained with CD 34 (arrow) in (subgp A, B) at 6^{th} dpi and 13^{th} dpt , respectively, **(c)** positive spindle cells stained with CD 34 (arrow) in subgp B at 5^{th} dpt.

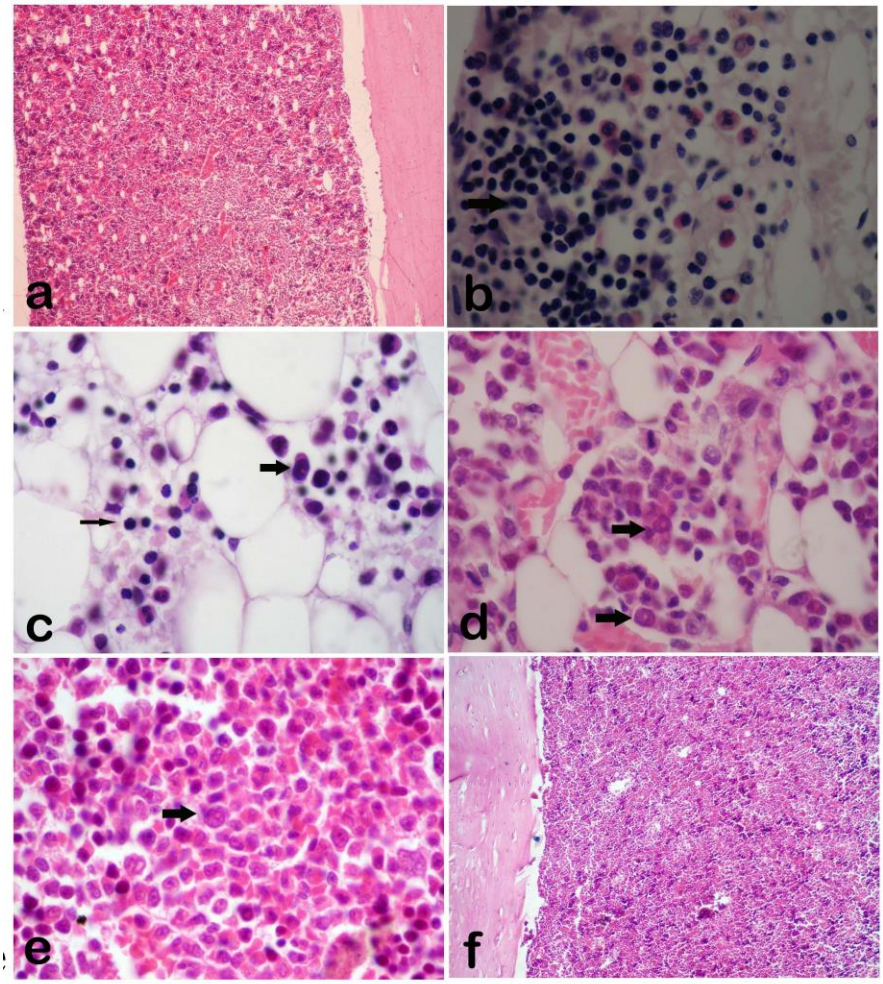

Figure 11: Photograph of Bone marrow, **(a)** normal structure of bone marrow in control group, **(b)** aggregation of blast leukemia cells (arrows), with **(c)** loss of cellularity and presence of myeloid dysplastic leukemia cells (neutrophils with ringed nucleus) (thick arrow) and myeloid blast cells (thin arrow) in subgp A at 6th and B at 1st dpi, **(d)** while at 5th dpt percentage of myeloid dysplastic leukemia cells with no myeloid blast cells, **(e)** at 10th dpt hypercellularity with reduction in myeloid dysplastic leukemic cells (arrow), **(f)** at 13th dpt normal architecture of bone marrow yield. **(H&E., x 80- 200)**

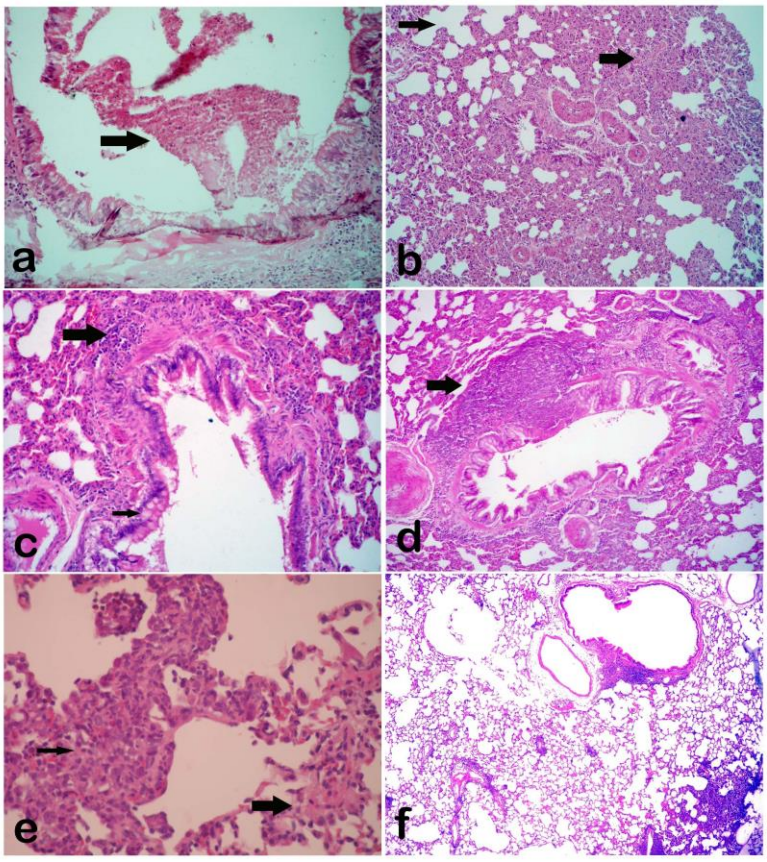

Figure 12: Lung stained with hematoxylin and eosin, (a) obstructed bronchioles with desquamated epithelial cells and neutrophilic debris inside the lumen (arrow**), (b)** interstitial pneumonia with aggregations of leukocytic cells besides, emphysematous areas in subgp A at 3th ,6th (dpi)**, (c)** bronchiolitis with metaplasia in the epithelial lining of bronchioles (thin arrow) , thickening in wall with edema and round cells infiltration (thick arrow) in subgp B at 1st dpt, **(d)** in addition to hyperplasia bronchial lymphoid tissue (arrow) at 5th dpt, **(e)** focal areas of hyperplasia in the septal cells (thin arrow) with increase of collagen fiber (thick arrow) in subgp B at 10th (dpt) **, (f)** regeneration in pulmonary tissues was detected in subgp B at 13th dpt. **(H&E., x 80-200)**