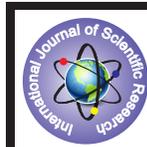


## Assessing the Role of Wheatgrass Supplementation in Rectification of Anemia: An *in-vitro* Approach



### Biotechnology

**KEYWORDS :** Anemia, Haematopoiesis, Wheat grass juice, EPO, Interleukins, CFUe

\* Pragnesh Banker

Ph.D. student, Dept. of Applied Sciences, Jodhpur National University, Jodhpur, Rajasthan  
\* is correspondent author

Dr. Rakesh Rawal

SSO and Head, Div. of Medicinal Chemistry & Pharmacogenomics, Dept. of Cancer Biology, The Gujarat Cancer & Research Institute, Ahmedabad, Gujarat.

### ABSTRACT

Anemia is a condition in which the body does not have enough healthy red blood cells which provides oxygen to body tissue. Many therapeutic options are available to treat anemia including supplementation of vitamins (Folic acid/vit-B12), Iron and phytopharmaceuticals e.g. wheatgrass juice and capsules. rEPO infusion in patients with anemia due to chronic disease including cancer is a routine practice when nutritional deficiencies are not identified as causative factors. In present study we scientifically evaluated the role of wheatgrass juice supplementation in the rectification of anemia. To substantiate the fact we developed an *in-vitro* model using mononuclear cells (MNC) derived from umbilical cord blood (UCB) and bone marrow of anemic patients other than cancer. Wheatgrass juice was supplemented in healthy volunteers of both sexes for 2 weeks. The circulating levels of various interleukins (1, 3 & 6), TNF- $\alpha$ , GM-CSF & EPO were estimated before and after supplementation. It was observed that IL-1, TNF- $\alpha$  showed decreasing trend while IL-3, IL-6 and GM-CSF showed increasing trend in supplemented sera as compared to unsupplemented sera. However EPO level remain unchanged or comparable between both the groups. The role of WGJ supplementation on CFU-e induction in HSC population was verified *in-vitro*. Cells were grown *in-vitro* on Methylcellulose media without EPO in presence of supplemented and un-supplemented sera. Development of higher CFU-e in supplemented sera generated evidence that secondary metabolites, interleukins and GM-CSF released as a result of wheatgrass juice metabolism directly or indirectly induce erythroid lineage in the progenitors independent of erythropoietin.

### Introduction

A biological process in which new blood cells are formed, and are usually taking place in bone marrow. Word origin from ancient Greek haema – Blood + poiesis – to make. Haematopoiesis is the process of production, multiplication, and specialization of blood cells in the bone marrow. All cellular component of the blood came from haemopoetic stem cells (HSCs) situated within the marrow. They are ultimately liable for the constant renewal of blood—the production of billions of new blood cells every day. Haematopoiesis begins with the most basic blood cell, the stem cell or “PHSC (pluripotent hematopoietic stem cell)”.

Anemia is a condition in which the body does not have enough healthy red blood cells which provides oxygen to body tissue. [1] Anemia due to chronic malignant disease is multifactorial and a common complication which may occurs as a direct effect of neoplasm. Notable among these are solid tumor malignancies, such as prostate and breast cancer that invades the marrow. Often overlooked as factors including anemia, these malignancies produce a desmoids or fibrotic reaction, with intersected marrow fibrosis that lead to alteration of marrow space and sinusoidal matrix. This could have an effect on the orderly release of mature blood cells from bone marrow and can produce a leukoerythroblastic picture with immature red cells and early myeloid white cells seen in peripheral blood.[2] In several cancer patients the causative mechanism of anemia is incompletely defined; therefore the term “anemia of chronic disease” is used. Defective iron utilization, the hallmark of anemia of chronic illness, is common among patient suffering from anemia due to malignancy.

In the past few years, various new approaches for the treatment of anemia have been introduced such as stem cell or bone marrow transplantation, transfusion of young red blood cells (neocytes), maintenance of a higher pre-transfusion hemoglobin level, new iron chelators and the use of drugs such as hydroxyurea, 5-AzaC and butyrate compounds to elevate foetal hemoglobin (HbF) level. Recent data from Indian laboratory have shown that wheatgrass has been shown to be effectiveness in thalassemia major in children.[3] Wheatgrass has been advocated for the treatment of various ailments such as high blood pressure, cancers, obesity, diabetes, gastritis ulcers, anemia, asthma and eczema however; the mechanism of effectiveness could not be demonstrated. Wheatgrass is a rich source of chlorophyll, various minerals like iron, magnesium, calcium, phosphorus, anti-

oxidants like beta carotene, vitamins, amino acids and insoluble dietary fibers. There is a prevailing myth that Chlorophyll and hemoglobin have similarity in structure and hence, it may be responsible for its effectiveness in rectification of anemia.

The colony forming cell (CFC) assay, also referred to as the methylcellulose assay, is an *in vitro* assay used in the study of hematopoietic stem cells. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation. The colonies formed can be enumerated and characterized according to their unique morphology.

The present study is undertaken to assess the medicinal value of wheat grass juice supplementation using an *in-vitro* model consisting of mono nuclear cells (MNCs) derived from human umbilical cord blood (h-UCB) and bone marrow (BM) from non malignant anemic patients.

### Materials & Methods

#### ELISA Assay

Serum Interleukin (IL1, 3, 6), EPO, GMCSF, TNF -  $\alpha$  levels were estimated using ELISA assay (R&D systems, USA) following manufacturer’s instructions. All assays were kept in duplicate.

#### CFU-E

##### Preparation of Mononuclear Cells:

UCB was collected in Heparinized Vacutainers after obtaining informed consent of donor (mother). Leftover bone marrow samples were collected from patients for anemia workup in Heparinized tubes filled with 2/3<sup>rd</sup> RPMI media. Immediately the samples were mixed gently to prevent clotting. Fifteen millilitre of UCB was diluted with equal volume of HBSS before proceeding to Ficoll-hypaque gradient centrifugation. The diluted UCB samples were overlaid on 15ml ficoll solution in a 50 ml sterile centrifuge tubes. Similarly the diluted bone marrow samples were overlaid on 5 ml of sterile Ficoll Plus in a 15ml sterile centrifuge tube. Tubes were centrifuged at 400 x g for 20 minutes with the brake off. MNCs were carefully harvested from the interface between the Ficoll-Paque PLUS and sample buffer using a sterile Pasteur pipette. The cells were transferred to sterile centrifuge tubes, washed with an equal volume of HBSS and centrifuged again for 10 minutes at 400g to remove the residual Ficoll-Paque PLUS. The cells were pooled together and washed for second time with large volume of HBSS.

**Methylcellulose Assay**

Aliquots of Methylcellulose-based Media and Cell Resuspension Solution were thawed at room temperature for approximately 30 minutes without disturbance. While the aliquots are thawing, the mononuclear cells were resuspended in 10 ml (or other appropriate volume) of IMDM and count. Around 5000-25000 cells were plated per 35 mm petri dish. The stock cell number is approximately 10x the final number needed for the experiment. The methylcellulose concentration in the final cell mixture should be approximately 1.27%. The vial was gently vortexed to thoroughly mix cells with the media. The tubes were incubated for 10min at RT to allow the air bubbles to escape. Group-I was supplemented with Day-0 sera and Group-II with day 14 sera. 1.1 mL of the final cell mixture was added to a 35 mm culture plate using a 3 mL syringe fitted with a 16 gauge needle. The media was spreaded evenly by gently rotating the plate. Two sample plates were placed along with an uncovered plate containing 3-4 mL sterile water in a 100 mm culture plate to maintain the humidity necessary for colony development. The cells incubated for 21 days at 37 °C and 5% CO<sub>2</sub> and monitored on day 3, 7, 14 and 21.

**Colony Scoring**

The colonies were identified and scored at the end of the incubation period using an inverted microscope and a scoring grid.

**Counting Criteria**

Colonies consisting of at least 20-40 cells are counted as positive.

**Colony validation**

Colonies were stained with 0.4% benzidine solution for eryth-

roid cells. Positive cells stained dark brown. Single and unstained colonies were not counted.

**Results**

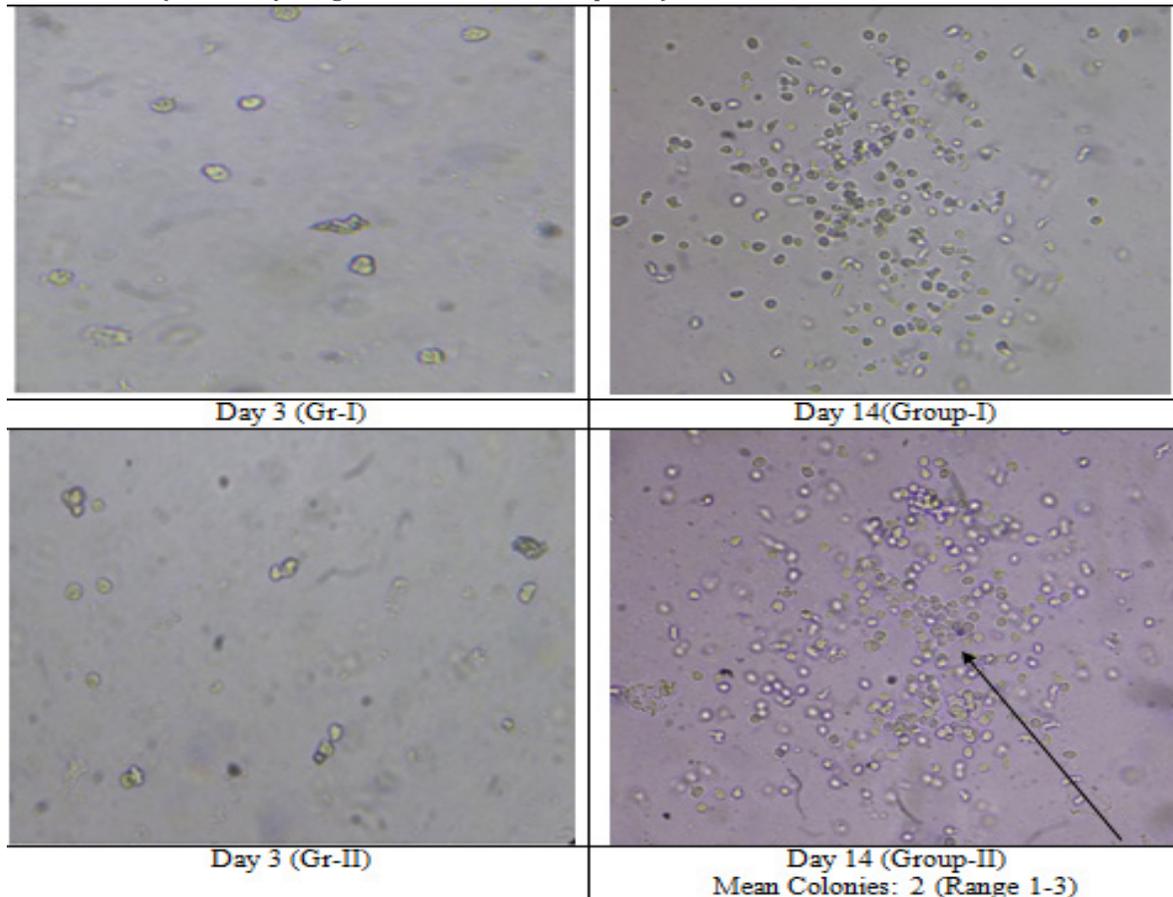
**Table-1: Result of Cytokine study in Healthy Volunteers by ELISA method**

	Before Supp	After Supp	Statistical analysis 't test p value'
EPO	10.17± 0.70 mIU/mL	8.95±0.95 mIU/mL	0.08↓(NS)
TNF-α	50.43±3.53 pg/mL	35.68±1.80 pg/mL	<0.001↓
Interleukin-1	1.86±0.04 pg/mL	1.78±0.04 pg/mL	0.062↓(NS)
Interleukin-3	30.47±4.14 pg/mL	94.28±13.09 pg/mL	<0.001↑
Interleukin-6	18.45±3.26 pg/mL	158.57±10.20 pg/mL	<0.001↑
GM-CSF	12.2±0.13 pg/mL	18.3±0.21 pg/mL	0.02↑

The circulating levels of various interleukins (1, 3 & 6), TNF-α, GM-CSF & EPO were estimated before and after supplementation. It was observed that IL-1, TNF- α showed decreasing trend while IL3, IL-6 and GM-CSF showed increasing trend in supplemented sera as compared to unsupplemented sera. However EPO level remain unchanged or comparable between both the groups.

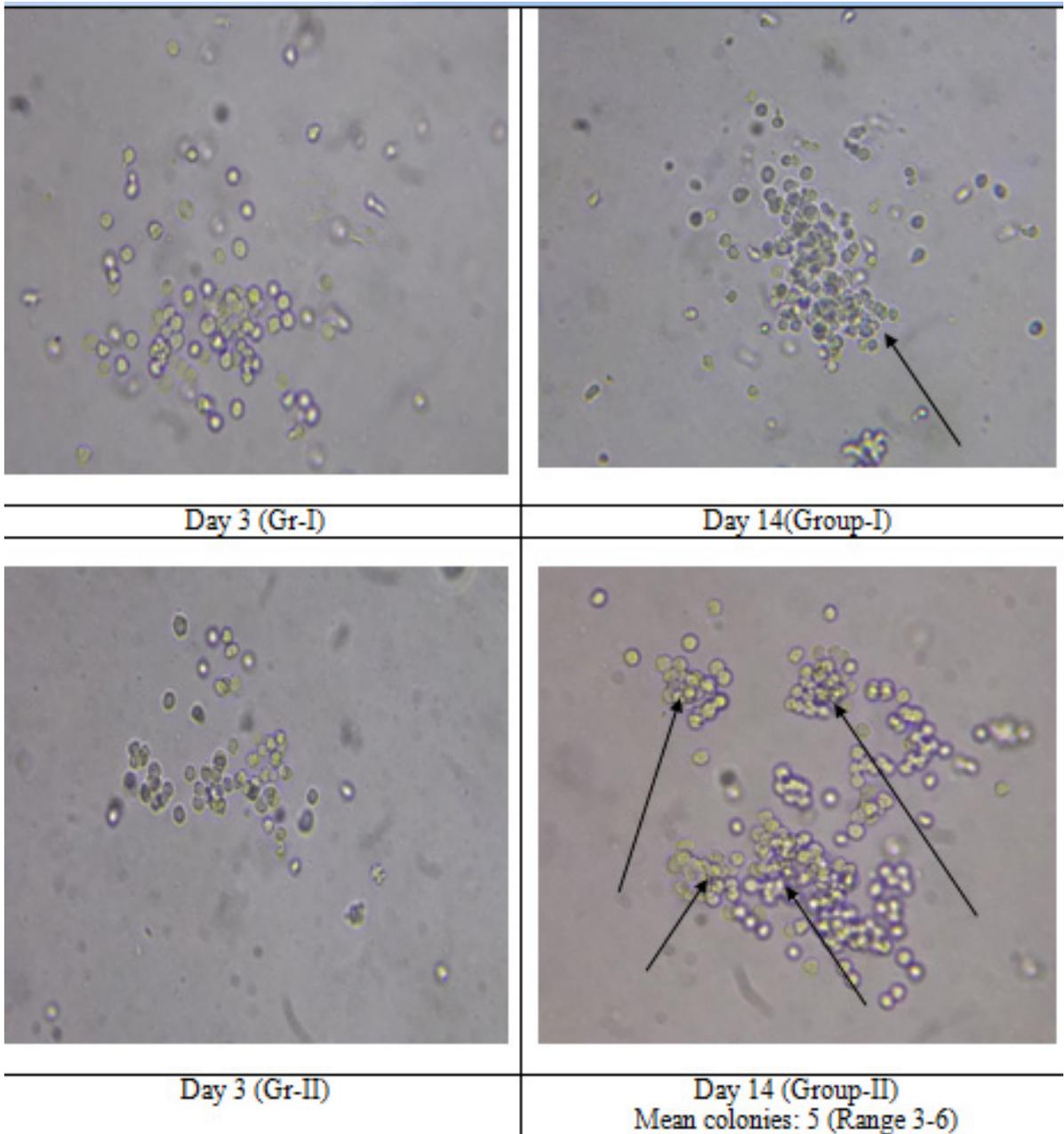
**CFU-E Assay**

**Picture 1: SET-I (CFU-e Assay using UCB derived MNC in Group-I & II)**



In set-I MNC derived from UCB were incubated with day0 (Group-I) and day14 (Group-II) serum for a period of 21days (3week) for CFU assay. Microscopic pictures shows presence of dividing cells in day 3 and increased cellularity at day 14. However no definite CFUs are observed in group-II till day 14 with occasional CFU in group-II (indicated by arrow).

Picture 2: SET-I (CFU-E Assay using BM derived HSC in Group-I & II)



In set-II for CFU assay MNC derived from anemic patient BM were incubated with group-I and II sera for a period of 21 days (3 weeks). Microscopic pictures show presence of dividing cells in day 3 and increased cellularity at day 14. Occasional CFUs are observed in group-I till day 14 however in group-II 4-5 CFUs were noted in each field (indicated by arrow). Result is suggestive of the role of supplemented sera in developing CFUs in anemic bone marrow but not in control UCB derived HSC which only showed increased cell number.

**Discussion**

All cellular blood elements are derived from hematopoietic stem cells residing within the medulla of the bone (bone marrow) and have the unique ability to give rise to all of the various mature blood cell types and tissues. HSCs are self-renewing cells: when they proliferate, at least some of their daughter cells remain as HSCs, so the pool of stem cells doesn't become depleted. [4] The other daughters of HSCs (myeloid and lymphoid progenitor cells), however can commit to any of the alternative differentiation pathways that lead to the production of one or more specific types of blood cells, but cannot self-renew. Development of blood cells is regulated by numerous growth factors and cytokines released by stromal cells, as well as mature leukocytes and T cells within the bone marrow. The specific regulatory functions of T cells and their role in hematopoiesis are to

be yet elucidated. [5]

In vitro culture of haematopoietic cells has provided some surprising insight into humoral regulation of haematopoietic cell growth. Every stage of haematopoiesis is subject to strict regulatory mechanisms involving humoral modulators. These factors called haematopoietins are a family of polypeptide hormones that specifically regulate the proliferation and differentiation of stem cells giving rise to erythrocytes, granulocytes, monocytes, megacaryocytes, and T and B lymphocytes. Mixed colonies consisting of elements of all haematopoietic lineages can be derived from pluripotent progenitors in vitro. Erythropoietin is the primary regulator of the later stages in erythropoiesis, whereas factors with burst-promoting activity or erythroid-potentiating activity stimulate the growth of the additional primitive eryth-

roid cells. The in vitro proliferation and differentiation of granulocytic and macrophage cells depends on the stimulation by a granulocyte-macrophage colony-stimulating factor.

The findings from the present study support the hypothesis that EPO independent erythropoiesis do exists as reported earlier in polycythemia vera mediated by transcription factor NF-E2 [6]. Correa PN reported earlier that IGF-1 could also stimulate colony formation by CFU-E from adult bone marrow cells in a EPO and serum-free ("SF") medium.[7] Erythropoietic stimulation via mechanisms which do not involve EPO receptors would have specific advantages in underproduction anemias such as the anemias of myelofibrosis, thalassemias, and marrow failure syndromes which are characterized by endogenous EPO concentrations that are elevated but ineffective.[8]

Supplementation of wheatgrass juice to healthy volunteers to assess its efficacy as rectifier of anemia using in vitro model is a unique approach. The study revealed variations in serum level of IL-1, 3, 6, TNF- $\alpha$  and GM-CSF due to WGJ supplementation.

Increased cellularity in UCB derived HSC and increased CFU-e in anemic bone marrow derived MNCs due to WGJ supplementation suggestive of differential regulation of division and differentiation pathway in normal and anemic condition. The increased colony formation in supplemented group might be due to elevated level of IL-3, 6 and GM-CSF acting in a coordinated way. A further in depth study is warranted to investigate the differential expression of genes involved in hematopoiesis in both the groups before establishing the role of WGJ supplementation as anemia rectifier.

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