Bone marrow hematopoietic stem cells behavior with or without growth factors in trauma hemorrhagic shock

Manoj Kumar, Sanjeev Bhoi, Sujata Mohanty1, Vineet Kumar Kama2, D. N. Rao3, Pravas Mishra4, Sagar Galwankar5

ABSTRACT

Background: Hemorrhagic shock (HS) is the major leading cause of death after trauma. Up to 50% of early deaths are due to massive hemorrhage. Excessive release of pro-inflammatory cytokine and hypercatecholamine induces hematopoietic progenitor cells (HPCs) apoptosis, leading to multiorgan failure and death. However, still, result remains elusive for hematopoietic stem cells (HSCs) behavior in trauma HS (T/HS).

Objectives: Therefore, our aim was to evaluate the in vitro HSCs behavior with or without recombinant human erythropoietin (rhEPO), recombinant human granulocyte macrophage-colony-stimulating factor (rhGM-CSF), recombinant human interleukin-3 (rhIL-3) alone, and combination with rhEPO + rhGM-CSF + rhIL-3 (EG3) in T/HS patients.

Methodology: Bone marrow (BM) aspirates (n = 14) were collected from T/HS patients, those survived on day 3. BM cells were cultured for HPCs: Colony-forming unit-erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), and colony-forming unit-granulocyte, monocyte/macrophage colonies growth. HPCs were counted with or without rhEPO, rhGM-CSF, rhIL-3 alone, and combination with EG3 in T/HS patients.

Results: BM HSCs growth significantly suppressed in T/HS when compared with control group (P < 0.05). In addition, CFU-E and BFU-E colony growth were increased with additional growth factor (AGF) (rhEPO, rhGM-CSF, and rhIL-3) as compared to baseline (without AGF) (P < 0.05).

Conclusion: Suppressed HPCs may be reactivated by addition of erythropoietin, GM-CSF, IL-3 alone and with combination in T/HS.

Key Words: Erythropoietin, granulocyte monocyte/macrophage-colony-stimulating factor, hematopoietic progenitor cells, interleukin-3 trauma-hemorrhagic shock

INTRODUCTION

Hemorrhagic shock (HS) is the leading cause of death in trauma. Mortality due to HS is approximately 50%.1 Fluid, blood component, and control of hemorrhage have been the cornerstone of management. Previous studies have shown that resuscitation with fluids and blood products induces reperfusion ischemia due to the production of reactive oxygen species and activation of immune cells.2 The elevated inflammatory cytokines contribute to the tissue damage. It also causes hematopoietic progenitor cells (HPCs: Colony-forming unit-erythroid [CFU-E], burst-forming unit-erythroid [BFU-E], colony-forming unit-granulocyte, monocyte/macrophage [CFU-GM]) apoptosis. It leads to multiorgan failure (MOF), following severe injuries and HS in human and animal models.3-4

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

Cite this article as: Kumar M, Bhoi S, Mohanty S, Kama VK, Rao DN, Mishra P, et al. Bone marrow hematopoietic stem cells behavior with or without growth factors in trauma hemorrhagic shock. Int J Crit Illn Inj Sci 2016;6:119-26.
HPCs apoptosis is a multifactorial process. Elevated levels of pro-inflammatory cytokine milieu and circulating catecholamine change the behavior of bone marrow (BM) microenvironment in severe trauma patients. Impaired HPCs are clinically associated with persistent anemia and are susceptible to infection, sepsis, and MOF.

EPO receptors are found on early BFU-E and late erythroid progenitor cells (EPCs) CFU-E. It helps proliferation and differentiation of EPC and nonhematopoietic tissue including central nervous system, endothelium, cardiac myocytes, and kidney. Erythropoietin (EPO) has anti-apoptosis, neuroprotective, anti-inflammatory, and angiogenesis properties. Animal study demonstrated protection of renal function, liver and neuromuscular injury in pretreatment group (intravenous EPO 3 days before induction of HS) when compared pretreatment with placebo (intravenous phosphate buffer saline [PBS] 3 days before induction of HS). EPO accelerated healing process after tibiofibular fractures in humans. Livingston et al. studied behavior of peripheral and BM HPCs growth at various time intervals. Suppressed HPCs growths were observed without reactivation.

The effect of additional growth factors (AGFs), such as EPO, GM-CSF, and interleukin-3 (IL-3) alone and conjugation with EG3 (recombinant human erythropoietin [rhEPO] + recombinant human GM-CSF [rhGM-CSF] + recombinant human IL-3 [rhIL-3]) on HPCs growth, has been poorly understood in patients with trauma HS (T/HS). We hypothesized that in vitro BM function may be reactivated with AGF. Therefore, our aim was to study in vitro colony growth of BM HPCs with or without AGFs in patients with T/HS presenting emergency department (ED) of a level 1 trauma center.

**METHODOLOGY**

**Study subjects**
Trauma patients of either sex, age group >18 and <60 years, and systolic blood ≤90 mmHg presenting within 8 h of injury to the ED of trauma center were recruited in the study. Patients with more than 8 h of injury, death within 3 days of admission, and who had received fluid or blood before presentation to ED were excluded from the study. Patients with preexisting hematologic diseases, anemia, active HIV infection, and renal and liver failures were excluded. Informed consent was obtained from each donor according to the guidelines of the Institutional Ethical Committee (approved Reference number IEC/NP-278/2010) of All India Institute of Medical Sciences, New Delhi - 110 029, India.

**Sample collection**
Two milliliters BM samples were obtained from Figure 1. BM aspirates were obtained from the iliac crest/sternum and placed into preservative-free heparin. Samples

---

**Figure 1:** Recruitment of trauma-hemorrhagic shock patients. BM: Bone marrow, ISS: Injury severity score, SI: Shock index, APACHE II: Acute Physiology of Chronic Health Evaluation II, CFU-E: colony-forming unit-erythroid, CFU-GM: Colony-forming unit-granulocyte, macrophage, BFU-E: Burst-forming unit-erythroid
were obtained from the survivors on day 3, admitted in Intensive Care Unit (ICU) or ward. The timing of sampling was on day 3. Senior residents performed BM aspiration. Informed consent was obtained from patients or patients’ relatives, who fulfilled the inclusion criteria before the procedure. Samples were collected after filling the patient information sheet and patient information consent form (PICF) [Annexures 1 and 2].

**Patient management and treatment**

All patients were treated with as per the Advance Trauma Life Support guideline. Shock index (SI), Acute Physiology of Chronic Health Evaluation II (APACHE II), and injury severity score (ISS) were calculated.

**Definition**

**Shock index**

The SI is defined as heart rate divided by systolic blood pressure, with a normal range of 0.5–0.7 in healthy adults. It is helpful to grade the severity of hemodynamic derangement after admission.\(^6\)\(^7\) SI was recorded once on arrival in the ED.

**Acute Physiology of Chronic Health Evaluation II**

APACHE II method is used for predicting hospital mortality among critically ill patients. It was noted within 24 h of admission in an ICU. Integer score from 0 to 71 was computed based on several measurements. Higher scores correspond to severe illness and higher risk of death.\(^8\)

**Injury severity score**

The ISS is an anatomical scoring system that provides an overall score for patients with multiple injuries. Each injury was assigned an Abbreviated Injury Scale (AIS) score and allocated to one of six body regions (head, face, chest, abdomen, extremities including pelvis, and external injury). The highest AIS score in each body region was used. The three most severely injured body regions have their score squared and added together to produce the ISS score.\(^9\)

**Control group**

We needed a normal marrow as a control. It would be unethical to ask normal individuals to donate marrow. Hence, it was decided to use patients whose marrow function is normal as a control, but coming for BM aspiration due to any reason. Patients with ITP have normal marrow function. However, increased peripheral destruction of platelets usually occurs in the spleen. The BM is done to demonstrate normal function as diagnostic criteria for ITP. BM sample was placed into preservative-free heparin. Samples were stored at 4°C before performing HPCs culture. Details of the control group are shown in Figure 2.

**Hematopoietic progenitor cell cultures with or without additional growth factors**

BM mononuclear cells (BMMNCs) were isolated from collected BM sample using Ficoll-Paque™ PLUS (M/s GE Healthcare Bio-Sciences AB, Upppsala, Sweden) density gradient centrifugation. BMMNCs were diluted with PBS in ratio of 1:4 and layered on Ficoll-Paque™ PLUS (2:1) and centrifuged at 1800 rpm × 30 min with room temp. Resultant buffy layer was collected from the interface and washed thrice with PBS to avoid the contamination of undesirable cells. The BMMNCs were pellet down at 1800 rpm × 10 min. Finally, the cells were resuspended in 1 ml IMDM + 2% FBS and MethoCult (M/s Stem Cell Technologies, Canada) and mixed thoroughly and cells were counted by hemocytometer. BMMNCs (2 × 10^5) were plated in duplicate in Methylcellulose media (M/s Stem Cell Technologies, Canada) with or without AGFs (rhEPO: 2.0 4.0 and 6.0 U/ml, rhIL-3: 3 U/ml, rhGM-CSF: 6 U/ml) (all from M/s. Peprotech, USA) alone and conjugation with EG3 [EPO [1 U/ml] + GM-CSF [3 U/ml] + IL-3 [1.5 U/ml]]. Cultures were incubated at 37°C in 5% CO₂. CFU-E colonies were counted at day 7; CFU-GM and BFU-E colonies were counted at day 14.

**Morphological characteristics of colony-forming unit-erythroid, burst-forming unit-erythroid and colony-forming unit-granulocyte, monocyte/macrophage colonies**

CFU-E, BFU-E, and CFU-GM were identified in a colony formation assay by the specific morphology of the cells.

CFU-E produces 8–200 erythroblasts in 1–2 clusters. Colonies are weakly or nonhemoglobinized [Figure 3a]. BFU-E produces three or more clusters. Colonies are usually large, dense, and hemoglobinized [Figure 3c]. CFU-GM produces a colony containing at least 40 granulocytes (CFU-G), macrophages (CFU-M), or cells of both lineages (CFU-GM) [Figure 3b].\(^10\)\(^11\)
Statistical analysis

**Data analysis**

Categorical and continuous data were expressed in frequency (%) and mean ± SD/median (minimum, maximum). The associations between two categorical variables were seen using Chi-square/Fisher’s exact test. For nonnormally distributed data, Mann–Whitney U-test was done to test the differences between two independent groups. Friedman test followed by post hoc analysis was used to detect differences in treatments for more than two groups. P < 0.05 was considered statistically significant. All the statistical analyses were done using statistical software Stata 12.1, StataCorp LP, 4905 Lakeway Drive College Station, Texas 77845, USA.

**RESULTS**

**Study subjects**

We recruited seventy patients with T/HS, of which 58 patients were excluded. Patients with coagulation defect (32),[12] death before day 3 (24), and contaminated samples (2) were excluded. BM samples were collected from 14 patients. Age group was between 18 and 60 years. Twelve males and two females in study group and 13 males and 13 females in control group were recruited for the study. SI, APACHE II, and ISS scores were recorded. The patient profile is depicted in Table 1. Details of the patients and control group are shown in Figures 1 and 2.

**Hematopoietic progenitor cell growth**

BM CFU-E, BFU-E, and CFU-GM colony growth decreased in T/HS when compared with control group (CFU-E: 27 vs. 71, P < 0.05, BFU-E: 11 vs. 88, P < 0.01, CFU-GM: 5 vs. 34, P < 0.05) [Table 2].

**Hematopoietic progenitor growth with or without additional growth factor**

CFU-E and BFU-E colony increased when added with AGF at different doses (rhEPO: 2.0, 4.0, 6.0 U/ml, rhIL-3: 3 U/ml, rhGM-CSF 6.0 U/ml) when compared with baseline control (without AGF), P < 0.05. CFU-E and BFU-E colony growth increased with 2.0 U/ml rhEPO (CFU-E: 64 vs. 27, P < 0.03, BFU-E: 17 vs. 11, P < 0.01) and with 3 U/ml rhIL-3 (CFU-E: 47 vs. 27, P < 0.01, BFU-E: 20 vs. 11, P < 0.01) [Table 3]. In addition, we explored the additive effect of EG3 (EPO + GM-CSF + IL-3) on HPCs colony growth. We found CFU-E (121 vs. 27, P < 0.05) colonies increased when compared with baseline control (CFU-E: 121 vs. 27, P < 0.01) [Table 3].

**DISCUSSION**

The present study indicates altered HPCs growth within BM compartment. It showed suppression of HPCs growth (CFU-E: 27 vs. 71, BFU-E: 11 vs. 88, CFU-GM: 5 vs. 34) when compared with control group. Livingston et al. observed suppressed BM progenitor cell growth in severely injured patients when compared to normal volunteer (28 ± 31 vs. 247 ± 24).[14] Table 4 compares the present study and Livingston et al. study for HPCs growth. Dysfunction of HPCs is a multifactorial process. HS induces elevated levels of inflammatory cytokines and circulating catecholamine which leads to HPCs apoptosis. BM dysfunction may lead to persistent anemia and risk of susceptibility to infection and sepsis.[4,15]
Kumar, et al.: Can be used growth factors for stimulation of hematopoietic progenitor cells in trauma hemorrhagic shock patients?

In addition, recent studies have reported that GM-CSF, G-CSF, and IL-3 stimulated BFU-E, CFU-GM, and CFU-Mix increased by 5.3-fold with perfusion cultures following T/HS patients.

Hematopoietic stem cells (HSCs) are derived from mesoderm. Previous studies demonstrated that HSCs have regeneration capacities and committed to multipotent, oligopotent, and unipotent progenitors. HSCs self-renewal is thought to occur in the stem cell niche.[14] HPCs microenvironment is controlled by a complex interplay between intrinsic signals surrounded by BM microenvironment.[12,13] Dysregulation of this balance can lead to BM dysfunction, which leads to hematopoietic and myeloproliferative disorder.[15] The present study reported impaired BM erythropoiesis in T/HS patients.

BM failure observed after trauma may be due to the lack of pro-regulatory cytokines within the BM microenvironment.[16] Moore et al. found decreased production of myelid colony-stimulating factors by monocytes from severely injured patients.[16] Inhibitory cytokines for hematopoiesis such as transforming growth factor-beta 1[27] have been reported to be elevated following severe trauma,[18] and it is possible that the BM failure observed is due to an overexpression of negative hematopoietic regulators.[17,18]

Erythropoietin induces erythropoiesis by promoting proliferation and differentiation of EPCs through the CFU-E. IL-3 and GM-CSF promote proliferation and differentiation of EPC.[19] There is a paucity of literature regarding the reactivation of BM dysfunction with AGFs (AGF, e.g. EPO, GM-CSF, and IL-3) alone and combination with EG3 following T/HS patients. However, we thought suppressed HPCs in T/HS may be reactivated by AGF alone and conjugation with EG3.

In this study, we used three growth factors, rhEPO, rhGM-CSF, rhIL-3 alone and combination of EG3 for stimulation and differentiation of HSCs in the BM.

Previous studies demonstrated the potential role of growth factor (rhEPO, rhIL-3, rhGM-CSF) in proliferation and differentiation of HSCs in BM.[20] In addition, IL-3 alone and in conjunction with IL-6, EPO, and GM-CSF stimulate proliferation of all cells in the myeloid lineage (granulocytes, monocytes, and dendritic cells) in murine model.[20,21] Wang et al. demonstrated that IL-3 and steel cell factor (SCF) have synergistic effect with EPO on the proliferation, differentiation, and apoptosis of EPCs in mice model. Furthermore, IL-3, EPO, and SCF were reported to act as anti-apoptotic, thereby inhibiting Bcl-2 family, such as Bcl-2 and Bcl-xl.[22]

The number of HPCs CFU-GM is increased by 18-fold and CFU-Mix increased by 5.3-fold with perfusion cultures with combination of SCF + IL-3 + IL-6 (S36). McNiece et al. reported that GM-CSF, G-CSF, and IL-3 stimulated distinct populations of GM-colony-forming cells.[24] To the...
best of our knowledge, there are no data available with AGF and conjugation with EG3 on patients with T/HS. However, few studies have reported in vitro BM HPCs increased with various growth factors (IL-3, GM-CSF, EPO, SCF, and G-CSF) in nontrauma patients.\[12\] The present study analyzed in vitro BM HPCs response to stimulation with AGF (EPO: 2 U/ml, IL-3: 3 U/ml) in T/HS patients. Previous in vitro studies reported synergistic interaction of hematopoietic growth factors.

In vivo animal studies demonstrated synergistic interactions with administration of various combinations such as GM-CSF, IL-3 with CSF-1, and GM-CSF with IL-3.\[25\] The present study suggests that the combination of EG3 is synergistic for in vitro CFU-E colony growth in T/HS patients.

Our study has some limitations that should be acknowledged. There was no sequential sampling for HSCs growth during the course of illness injury. Several factors such as gender, sex steroids, and genetic polymorphisms may have influenced inflammatory cytokine levels, which could not be controlled due to the design of the study.\[31\] In this small sample of 12 patients, it is very difficult to determine which factors are the leading causes for BM failure. However, our results demonstrated that BM dysfunction may be reversible with AGF (rhEPO 2 U/ml, rhIL-3: 3 U/ml) alone and conjugation with EG3 in T/HS patients. The use of growth factors may help shorten the recovery of suppressed HPCs and reduced blood transfusion. Further studies with large sample sized clinical trial with growth factors in T/HS in animal model may provide further insight.

CONCLUSION

Suppressed HPCs may be reactivated by addition of erythropoietin, GM-colony-stimulating factor, IL-3 alone and with combination in T/HS.

Financial support and sponsorship

This study was financially supported by Indian Council of Medical Research (ICMR).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Kumar M, Bhoi S. Does erythropoietin reactivate bone marrow dysfunction in trauma hemorrhagic shock? Int J Crit Illn Inj Sci 2015;5:230-1.
2. Finfer S, Liu B, Taylor C, Bellomo R, Billot L, Cook D, et al. Resuscitation fluid use in critically ill adults: An international cross-sectional study in 391 intensive care units. Crit Care 2010;14:R185.
3. Sifri ZC, Kaiser VL, Ananthakrishnan P, Wang L, Mohr AM, Hauser CJ, et al. Bone marrow failure in male rats following trauma/hemorrhagic shock (T/HS) is mediated by mesenteric lymph and modulated by castration. Shock 2006;25:12-6.
4. Livingston DH, Anjaria D, Wu J, Hauser CJ, Chang V, Deitch EA, et al. Bone marrow failure following severe injury in humans. Ann Surg 2003;238:748-53.
5. Fonseca RB, Mohr AM, Wang L, Sifri ZC, Rameshwar P, Livingston DH. The impact of a hypercatecholamine state on erythropoiesis following severe injury and the role of IL-6. J Trauma 2005;59:884-9.
6. Roumen RM, Hendriks T, Wevers RA, Goris JA. Intestinal permeability after severe trauma and hemorrhagic shock is increased without relation to septic complications. Arch Surg 1993;128:453-7.
7. Allgöwer M, Burri C. Shock index. Dtsch Med Wochenschr 1967;92:1947-50.
8. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: A severity of disease classification system. Crit Care Med 1985;13:818-29.
9. Baker SP, O’Neil B, Haddon W Jr, Long WB. The injury severity score: A method for describing patients with multiple injuries and evaluating emergency care. J Trauma 1974;14:187-96.
10. Iscove NN, Sieber F. Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. Exp Hematol 1975;3:32-43.
11. Nakao K, Kosaka M, Saito S. Effects of erythropoietin differentiation factor (EDF) on proliferation and differentiation of human hematopoietic progenitors. Exp Hematol 1991;19:1090-5.
12. Patel P, Davidson JC, Nikolic B, Salazar GM, Schwartzberg LS, Walker TG, et al. Consensus guidelines for perioperative management of coagulation status and hemostasis risk in percutaneous image-guided interventions. J Vasc Interv Radiol 2012;23:727-36.
13. Robinson Y, Hostmann A, Matenov A, Ertel W, Oberholzer A. Erythropoiesis in multiply injured patients. J Trauma 2006;61:1285-91.
14. Hematopoietic Stem Cells. Stem Cell Information. National Institutes of Health, U.S. Department of Health and Human Services; 2011. http://stemcells.nih.gov/info/basics/Pages/Default.aspx [Last accessed on 2013 Nov 09].
15. Robinson Y, Matenov A, Tschöke SK, Weimann A, Oberholzer A, Ertel W, et al. Impaired erythropoiesis after haemorrhagic shock in mice is associated with erythroid progenitor apoptosis in vivo. Acta Anaesthesiol Scand 2008;52:605-13.
16. Moore FA, Peterson VM, Moore EE, Rundus C, Poggetti R. Inadequate granulopoiesis after major torso trauma: A hematopoietic regulatory paradox. Surgery 1990;108:667-74.
17. Meert KL, Ofenstein JP, Genyee C, Sarnaik AP, Kaplan J. Elevated transforming growth factor-beta concentration correlates with posttrauma immunosuppression. J Trauma 1996;40:901-6.
18. Murohashi I, Endho K, Nishida S, Yoshida S, Jinnai I, Bessho M, et al. Differential effects of TGF-beta 1 on normal and leukemic human hematopoietic cell proliferation. Exp Hematol 1995;23:970-7.
19. Elliott S, Sinclair AM. The effect of erythropoietin on normal and neoplastic cells. Biologics 2012;6:163-89.
20. Yang YC, Ciarietta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, et al. Human IL-3 (multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell 1986;47:3-10.
21. Francisco-Cruz A, Aguilar-Santelises M, Ramos-Espinosa O, Mata-Espinosa D, Marquina-Castillo B, Barrios-Payan J, et al. Granulocyte-macrophage colony-stimulating factor: Not just another hematopoietic growth factor. Med Oncol 1995;23:970-7.
22. Wang J, Tang ZY, Ka W, Sun D, Yao W, Wen Z, et al. Synergistic effect of cytokines EPO, IL-3 and SCF on the proliferation, differentiation and apoptosis of erythroid progenitor cells. Clin Hemorheol Microcirc 2007;37:291-9.
23. Koller MR, Bender JG, Miller WP, Papoutsakis ET. Expansion of primitive human hematopoietic progenitors in a perfusion bioreactor system with IL-3, IL-6, and stem cell factor. Biotechnology (N Y) 1993;11:358-63.
24. Nicewicz I, Andrews R, Stewart M, Clark S, Boone T, Quesenberry P. Action of interleukin-3, G-CSF, and GM-CSF on highly enriched human hematopoietic progenitor cells: synergistic interaction of GM-CSF plus G-CSF. Blood 1989;74:110-4.
25. Kobari I, Giarratana MC, Poloni A, Firan H, Labopin M, Gorin NC,
Kumar, et al.: Can be used growth factors for stimulation of hematopoietic progenitor cells in trauma hemorrhagic shock patients?

et al. Flt 3 ligand, MGDF, Epo and G-CSF enhance ex vivo expansion of hematopoietic cell compartments in the presence of SCF, IL-3 and IL-6. Bone Marrow Transplant 1998;21:759-67.
26. Vassiliou I, Lolis E, Nastos C, Tympa A, Theodosopoulos T, Dafnios N, et al. The combined effect of erythropoietin and granulocyte macrophage colony stimulating factor on liver regeneration after major hepatectomy in rats. World J Surg Oncol 2010;8:57.
27. Singh VK, Newman VL, Seed TM. Colony-stimulating factors for the treatment of the hematopoietic component of the acute radiation syndrome (H-ARS): a review. Cytokine 2015;71:22-37.
28. Lemoli RM, Fogli M, Fortuna A, Motta MR, Rizzi S, Benini C, et al. Interleukin-11 stimulates the proliferation of human hematopoietic CD34 and CD34 CD33-DR- cells and synergizes with stem cell factor, interleukin-3, and granulocyte-macrophage colony-stimulating factor. Exp Hematol 1993;21:1668-72.
29. Broxmeyer HE, Williams DE, Hangoc G, Cooper S, Gillis S, Shadduck RK, et al. Synergistic myelopoietic actions in vivo after administration to mice of combinations of purified natural murine colony-stimulating factor 1, recombinant murine interleukin 3, and recombinant murine granulocyte/macrophage colony-stimulating factor. Proc Natl Acad Sci U S A 1987;84:3871-5.
30. Donahue RE, Seehra J, Metzger M, Lefebvre D, Rock B, Carbone S, et al. Human IL-3 and GM-CSF act synergistically in stimulating hematopoiesis in primates. Science 1988;241:1820-3.
31. Mountford JC, Bunce CM, Hughes SV, Dryson MT, Webb D, Brown G, et al. Estrone potentiates myeloid cell differentiation: a role for 17 beta-hydroxysteroid dehydrogenase in modulating hemopoiesis. Exp Hematol 1999;27:451-60.
32. Hörner S, Pasternak G, Hehlmann R. A statistically significant sex difference in the number of colony-forming cells from human peripheral blood. Ann Hematol 1997;74:259-63.
33. Cohen M, Volpin G, Meir T, Klein E, Katz R, Assaf M, et al. Possible association of Toll-like receptor 9 polymorphisms with cytokine levels and posttraumatic symptoms in individuals with various types of orthopaedic trauma: Early findings. Injury 2013;44:1625-9.
34. Pervanidou P, Kolaitsis G, Charitaki S, Margeli A, Ferentinos S, Bakoula C, et al. Elevated morning serum interleukin (IL)-6 or evening salivary cortisol concentrations predict posttraumatic stress disorder in children and adolescents six months after a motor vehicle accident. Psychoneuroendocrinology 2007;32:991-9.
35. Newport DJ, Nemeroff CB. Neurobiology of posttraumatic stress disorder. Curr Opin Neurol 2000;10:211-8.

ANNEXURE 1

Patient information sheet (PIS)

i) Title of the project
- To study the Role of recombinant human erythropoietin in trauma victims with hemorrhagic shock: An in vitro approach through stem cell differentiation.

ii) Aims and methods of the research
- To evaluate the effect of recombinant human erythropoietin (rhEPO) on in vitro hematopoietic progenitor cell growth/differentiation among hemorrhagic shock (HS) patients
- To assess the serum levels of EPO and Pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ, TNF-α) among HS patients
- To correlate the alone invitro readout parameters with clinical outcomes of HS patients.

iii) Expected duration of the subject participation
- Three years.

iv) The benefits to be expected from the research to the subject or to others
- Not required for subject, will provide information for improving treatment to future patients.

v) Any risk to the subject associated with the study
- No risk to the subject associated with the study.

vi) Maintenance of confidentiality of records
- All patient records will be kept confidential.

vii) Provision of free treatment for research related injury
- The study does not involve any risk to the patient.

viii) Compensation of subjects for disability or death resulting from such injury
- Does not arise.

ix) Freedom of individual to participate and to withdraw from research at any time. Without penalty or loss of benefits to which the subject would otherwise be entitled
- The subject will have the freedom to withdraw from research at any time.

x) Amount of blood sample to be taken should be mentioned in PIS in Tea Spoon Full
- 2 to 3 tea spoon

xi) Costs and source of investigations, disposables, implants and drugs/contrast media must be mentioned in the PIS
- Mentioned.

xii) Telephone number/contact number of the candidate and one of the investigators must be mentioned in the PIS
- Mentioned.

xiv) Self certification should be given that translation to vernacular is accurate
- Attached.
**ANNEXURE 2**

**Participant informed consent form (PICF)**

Protocol/Study number: ______________________

Participant identification number for this trial:

Title of project: To study the role of erythropoietin in trauma victims with hemorrhagic shock: In *In vitro* approach stem cell differentiation.

Name of Project investigator Dr. Sanjeev Bhoi

Tel.No(s) 9868397053

The contents of the information sheet dated that was provided have been read carefully by me/explained in detail to me, in a language that I comprehend, and I have fully understood the contents. I confirm that I have had the opportunity to ask questions.

The nature and purpose of the study and its potential risks/benefits and expected duration of the study, and other relevant details of the study have been explained to me in detail. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal right being affected.

I understand that the information collected about me from my participation in this research and sections of any of my medical notes may be looked at by responsible individuals from AIIMS. I give permission for these individuals to have access to my records.

I agree to take part in the above study.

(Signatures/Left Thumb Impression)

Place:-------------------

Name of the Participant: ________________________

Son/Daughter/Spouse of: ________________________

Complete postal address: ________________________

This is to certify that the above consent has been obtained in my presence.

(Signatures of the Principal Investigator)

Place:-------------------

1) Witness – 1  2) Witness – 2

(Signatures)

Name:-------------------

Address:-------------------

NB Three copies should be made, for (1) patient, (2) researcher, (2) Institution

(Investigators are advised to prepare the translation in simple understandable Hindi on their own).