

Recombinant human erythropoietin reduces plasminogen activator inhibitor and ameliorates pro-inflammatory responses following trauma

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ABSTRACT

Background and the purpose of the study: Besides its hematopoietic effects, erythropoietin (EPO) by mobilization of iron and modulation of some inflammatory cytokines has antioxidant and anti-inflammatory properties. The purpose of this study was to evaluate these effects of erythropoietin and its impact on organ function in traumatized patients.

Methods: Twenty-six ICU-admitted traumatized patients within 24 hrs after trauma were randomly assigned to the EPO (received EPO, 300 units/Kg/day) and Control (not received EPO) groups. The inflammatory biomarkers including Tumor Necrosis Factor alpha (TNF- α), Interleukin 1 (IL-1), Plasminogen Activator Inhibitor 1 (PAI-1) and Nitrotyrosine were recorded at the admission, 3, 6 and 9 days thereafter. Acute Physiology and Chronic Health Evaluation (APACHE II) and Sequential Organ Failure Assessment (SOFA) scores were also recorded.

Results: Among 12 patients (EPO group) TNF- α level at the day of 9 (P=0.046), and within EPO group at the days of 3 (P=0.026 ameliorate), 6 (P=0.016), and 9 (P=0.052) were significantly lowered. Level of IL-1 and PAI-1 decreased significantly at days of 3, 6 and 9 post intervention. Also there were significant differences between two groups in the SOFA score during three measured time intervals (the first, third and seventh days).

Conclusion: From the results of this study it seems that injection of erythrocyte stimulating agent is well tolerated and inhibits the inflammatory response and oxidative stress following trauma.

Keywords: Anti-inflammatory, Anti-oxidative, Recombinant Human Erythropoietin, SOFA, Trauma.

INTRODUCTION

According to WHO estimation, traumatic injuries are leading cause of morbidity and mortality throughout the world especially in underdeveloped countries (1). Among different reasons and mechanisms, volume loss and hypovolemic shock secondary to hemorrhage is one of the major causes of death of traumatized patients (1). Although fluid therapy and blood transfusion play an essential role in correction of blood loss but, transfusion has its drawback such as infection transmission, acid-base disequilibrium, hypothermia, electrolyte disturbance, deep vein thrombosis, and also decrease patient survival. Blood loss, nutritional deficiency, and endocrine, renal and hepatic insufficiencies are some of the reasons for anemia in traumatized and critically ill patients. Erythropoietin a secreted glycoprotein by kidney through augmenting of hematopoietic

remarkably improve anemia and blood oxygen supply in critically ill patients (2).

Production and release of free radicals following traumatic injury is one of the underlying mechanisms leading to cell injury and subsequent organ failure and it has been reported that excess free radicals overwhelmed antioxidant defense of body. Many research have been performed with antioxidant drugs including N-acetylcysteine (3), vitamin C (4), vitamin E (5) and even iron chelators (6) for prevention of oxidative stress following trauma, but results are not convincing. In addition to the obvious role of EPO for treatment of anemia, several clinical reports have shown that it has also antioxidant effects (7).

EPO by increasing hemoxygenase-1 within cells (7), degrade hem and release iron for subsequent utilization in hematopois. Metal ions especially

iron by production of powerful hydroxyl radicals play an important role in oxidative stress process and it seems that EPO mobilizes iron in blood cell production cycle and decrease oxidative stress. Moreover, increasing numbers of circulating young red blood cells indirectly reduce cellular oxidative stress (7). This study assesses the antioxidant and anti-inflammatory effects of EPO on key systemic inflammatory response syndrome (SIRS) mediators in traumatized patients.

MATERIAL AND METHODS

Study design and setting

This study was an open label randomized clinical trial (Registered under government ID number NCT00622934), conducted at Intensive Care Unit of "Sina" teaching hospital (Tehran University of Medical Sciences, Tehran, Iran). Traumatized patients within 24 hrs after trauma referring to the hospital from October 2008 to October 2009 were enrolled in the study. In all cases, informed consent was obtained from patients or their closest relatives. The study procedure and protocol were approved by the ethical committee of Tehran University of Medical Sciences and Health Services. Randomization was stratified by utilizing a computer-generated random numbers.

Study Population

All patients had been admitted to either a medical, surgical, or a medical/surgical ICU. Inclusion criteria were; staying in the ICU for 3 days, hematocrite less than 28%, and provision of signed informed consent. Exclusion criteria included age less than 18 years, renal failure with dialysis, new onset or uncontrolled seizures, acute burns, pregnancy or lactation, acute ischemic heart disease, acute gastrointestinal bleeding, prior treatment with EPO, participation in another research protocol, and expected ICU discharge within 48 hrs.

Methods

Patients were randomly assigned to two groups. In the EPO group (n=12), 300unit/kg of EPO (Pooyesh Darou-Poe tin®, Tehran, Iran) were injected subcutaneously and continued every other day for a total of 3 doses (days of 1, 3 and 5) and followed up for 10 days. Blood samples were collected from central catheter at the day of admission and 3, 6 and 9 days thereafter. All samples were spun for 15 min and plasma was stored at -80°C until the time of analysis.

The following transfusion guideline was considered for the study: no RBC transfusion if the hemoglobin level was at least 9 g/dl or the hematocrite concentration was at least 27%, unless there was a specific clinical indication (active bleeding, ischemia); RBC transfusion for a hemoglobin level

less than 9 g/dl or a hematocrite concentration less than 27% according to physician's judgment.

Patients' clinical and preclinical characteristics were recorded as follows: consciousness according to the Glasgow Coma Scale (GCS), saturation oxygen tension (SPO₂), blood urea nitrogen (BUN), creatinine (Cr), serum electrolytes including sodium, potassium, magnesium, calcium, phosphorus, blood sugar (BS), WBC count, platelet count, hemoglobin, hematocrite, arterial blood gas (ABG), body temperature (using a rectal probe) and blood pressure, pulse rate, respiratory rate 4 times a day or according to the physician's order. All measurements were performed at the admission, within the first 3 days, and at the days of 6 and 9 after admission. Patients also underwent Acute Physiology and Chronic Health Evaluation (APACHE) II scores (8), injury severity score (ISS) (9) based on data obtained within the first 24 hrs after ICU admission. Sequential Organ Failure Assessment (SOFA) (10) as for organ dysfunction was assessed in the first and third and seventh days of admission.

All participants were continuously monitored for their hemodynamic parameters. Following biomarkers were considered as the inflammatory reactants: C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Tumor Necrosis Factor alpha (TNF- α), interleukin 1 (IL-1), plasminogen activator Plasminogen Activator Inhibitor 1 (PAI-1) and nitro tyrosine test (for anti-oxidant power).

Co-morbidity factors were identified from the medical history including cardiac disease, chronic pulmonary disease, diabetes mellitus, hypertension, malignancy, peripheral vascular diseases, and embolic diseases.

Cytokine measurements

Inflammatory biomarkers concentrations in plasma were measured by using commercially available enzyme-linked immunosorbent assay kits as follows: TNF- α (Bender Med Systems ,Austria, Europe), IL-1 (Bender Med Systems ,Austria, Europe), PAI-1 (American diagnostic, Germany, Europe) and Nitrotyrosine (Hycult biotechnology b. v. , Netherlands, Europe) .

Statistical analyses

The frequency of all demographic and clinical characteristics was calculated. Independent two sample t test was used to compare the mean differences of the clinical and preclinical variables between and within groups compared to baseline. For non-parametric data, Mann-Whitney U test (a non-parametric two independent samples test) was used. The quantitative results are shown as mean (\pm standard deviation) or number (percent) if necessary. All statistical calculations were conducted using SPSS ver. 14. P and values <0.05 was considered significant.

Table 1: Baseline Characteristics.

| | Control group n=13 | Epo group n=12 | P Value |
|--------------------------------|--------------------|----------------|---------|
| Age, mean (SD), year | 39.33 (23.8) | 32.46 (13.5) | 0.667 |
| APACHE II score, mean (SD) | 19.6 | 19.7 | 0.43 |
| ISS | 13.5 | 13.7 | 0.39 |
| SOFA | 9.5 | 7.1 | 0.41 |
| Baseline inflammatory factors | | | |
| TNF- α (pg/ml) | 12.32 | 12.21 | 0.824 |
| IL1 (pg/ml) | 6.43 | 5.34 | 0.301 |
| PAI1 (ng/ml) | 62.07 | 65.75 | 0.520 |
| Nitro tyrosin (10^{-9} mol) | 10.7 | 6.15 | 0.299 |

Table 2: Comparison of changes in SOFA scores during days.

| | | Epo group | Control group | P value |
|------|-------|---------------|---------------|---------|
| SOFA | Day 1 | 7.1 \pm 0.5 | 9.5 \pm 0.7 | 0.006 |
| | Day3 | 5.8 \pm 0.5 | 8.8 \pm 0.8 | 0.002 |
| | Day7 | 5.3 \pm 0.5 | 8.9 \pm 0.9 | 0.001 |

RESULTS

Twelve EPO and 13 control patients with the mean age of 39.33 (\pm 23.8) and 32.46 (\pm 13.5) years respectively ($P=0.38$) underwent intervention. All recruited patients were men. Among 26 recruited patients, one patient was transferred to the neurosurgery department. ICU scores including APACHE II, and ISS scores were not significantly different between two groups [The relevant P values (and 95% confidence interval) were 0.43 (-2.31_-0.04), 0.39 (-4.02_1.64) and 0.41 (-0.63_1.47) respectively] (Table 1).

SOFA score was assessed in the first and third and seventh days, the patients who did not receive EPO had higher SOFA score (Table 2).

Patients' consciousness, body temperature, blood pressure, pulse rate (except for higher pulse rate within EPO group at the 6th day, ($P=0.042$)), respiratory rate, SPO₂, BUN, Cr, serum sodium, potassium, calcium & phosphorus, blood sugar, Hb and Hct were not significantly different between and within groups. The Mann-Whitney test showed similar results, except for Hct which was higher as a trend within the 9th day of ICU staying (P value for the day of 3, 6 and 9 were 0.06, 0.08 and 0.06 respectively).

Mann-Whitney U test showed that TNF- α level between groups at the day of 9 ($P=0.046$), and its level within EPO group at the days of 3 ($P=0.026$), 6 ($P=0.016$), and 9 ($P=0.052$) were significantly lower (Fig 1).

IL-1 was significantly lower at the days of 3 and 6 after treatment in EPO group [$P=0.051$ (95 CI: -0.006_3.03) and $P=0.005$ (95 CI: 0.89-4.23)] respectively

Moreover, Mann-Whitney U test indicated that IL-1

level at the day of 9 was also significantly lower in EPO group ($P=0.030$) (Fig 2).

The relevant independent samples t-test analysis about PAI-1 suggest that PAI-1 levels between groups and within EPO group significantly decreased from the day of 3 ($P=0.011$) toward the end of treatment ($P<0.001$). Mann-Whitney U test confirmed the same results ($P=0.008$ for the day of 3, and $P<0.001$ for the rest of days) (Fig 4).

The statistical analysis (independent samples t-test) proposed that nitro tyrosine levels were significantly lower in EPO than EPO Control groups at the days of 6 ($P=0.006$, 95 CI: 2.10-10.52) and 9 ($P=0.007$, 95 CI: 2.12-11.21). Applying the Mann-Whitney U test indicated that nitro tyrosine level was lowered significantly not only at the days of 6 and 9 (P value were 0.046 and 0.030 respectively) between groups, but also within EPO group at the same days (P value was 0.035 for the day of 6) (Fig 3).

DISCUSSION

EPO exerts humeral effects by means of its specific receptor (EPO-R) which does not exist in many tissues under normal conditions; but several metabolic stressors (like ischemia, hypoglycemia, etc) rapidly provoke up-regulation of the EPO-R which in turn leads to inhibition of production of pro-inflammatory cytokines like TNF- α , IL-1 β , IL-6, IL-8, PAF, etc (11). The cytoprotective mechanism of EPO is not completely understood but a probable explanation has been suggested (11).

Our recent investigations have revealed remarkable results on the role of cytokines such as IL, growth factors and oxidative stress mediators in the pathology or prognosis of many chronic and acute diseases and these issues led us to study the effect of

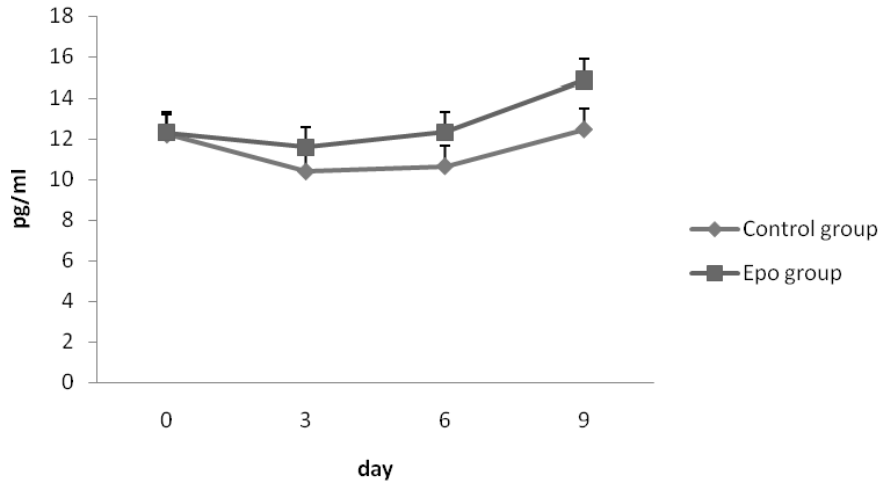


Figure 1: Changes in plasma concentrations of TNF- α . There were significant differences in TNF- α level between two groups at the day of 9 ($P=0.046$), and its level within EPO group at the days of 3 ($P=0.026$), 6 ($P=0.016$), and 9 ($P=0.052$) compared with baseline.

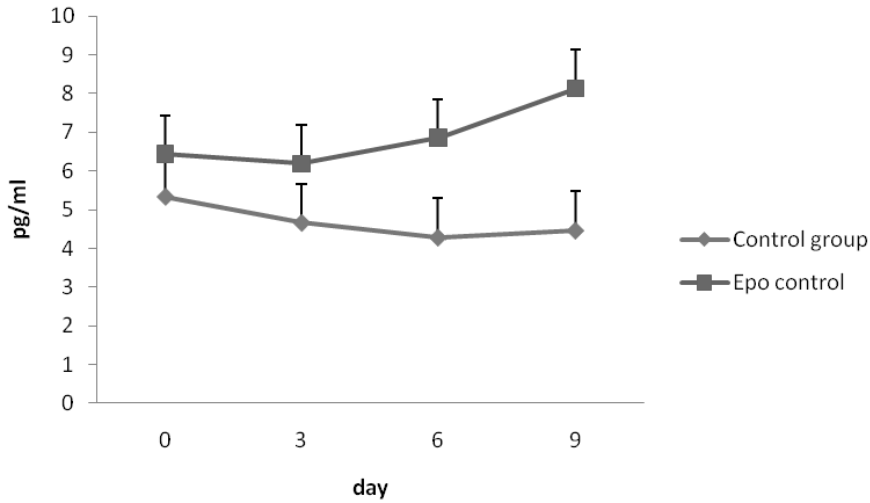


Figure 2: Changes in plasma concentrations of IL-1. There were significant differences in IL-1 between two groups at the days of 3 and 6 ($P=0.051$ and 0.005 respectively) and its level within EPO taken group at the day of 9 compared with baseline ($P=0.030$).

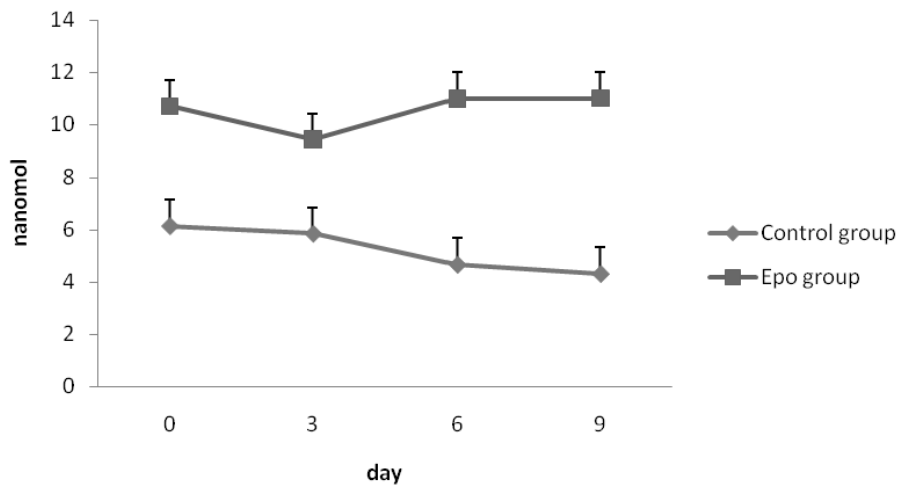


Figure 3: Changes in plasma concentrations of Nitro tyrosine. Nitro tyrosine levels were significantly lower in EPO group than Control group at the days of 6 and 9 ($P=0.006$ and 0.007 respectively)

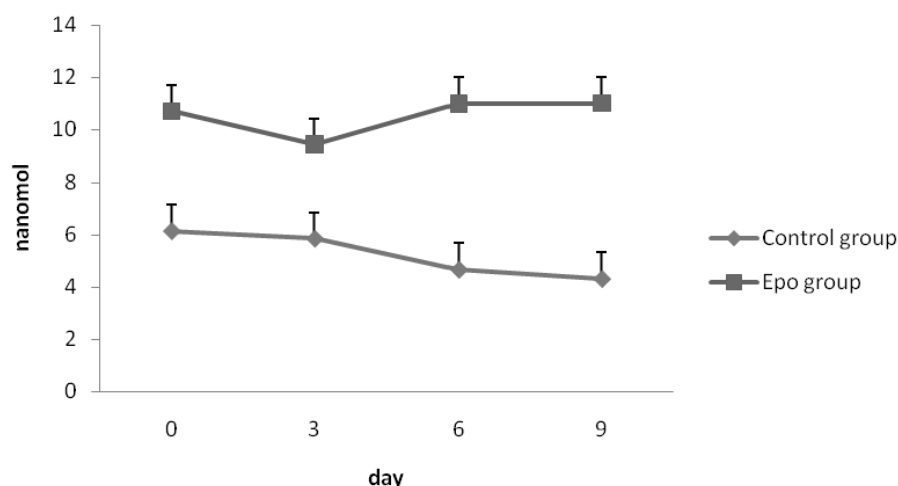


Figure 4: Changes in plasma concentrations of Plasminogen Activator Inhibitor 1 (PAI-1). There was significant decrease in plasma level of (PAI-1) in EPO group at the day of 3, 6 and 9 ($P=0.11$, <0.001 and <0.001 respectively).

EPO on these variables (12).

In the present study, the ICU scores including APACHE II, ISS showed insignificant discrepancy between the study groups; therefore further comparison of clinical and para-clinical (inflammatory biomarkers) were made between groups irrespective of the severity of trauma.

Results showed that IL-1 as an inflammatory reactant significantly decreased after administration of EPO for 3 days and this effect lasted to the 9th day after the treatment was started. Concerning the TNF- α , there was a remarkable decrease in the relevant levels after 9 days treatment with EPO in comparison with EPO control group; and TNF- α level also significantly decreased in the days of 3, 6 and 9 after treatment compared to the baseline level in the EPO group.

In agreement with reports that EPO has a variety of direct anti-inflammatory effects (13), results of this study showed that EPO significantly diminished synthesis of anti-inflammatory cytokines. This indicates that the observed inflammatory effects of EPO were not mediated by an increase in the principal regulatory cytokines, but were either cytokine independent or mediated by a different cytokine(s). Inhibition of apoptosis has been suggested as a fundamental mechanism of EPO-mediated tissue protection. However, in an invitro model it was found that EPO rescues only a small number of cytokine-producing cells from apoptosis (data not shown).

The cytokine inhibitory properties of EPO likely represent an important mechanism by which its tissue-protective effects are mediated and evidence suggests that cytokine-mediated cell injury plays an essential role for end-organ damage (14).

In accordance with findings of this study, it seems that PAI-1 was affected strongly and remarkably by EPO administration because its level fell significantly

from the third day after treatment and remained low during the study period. PAI-1 is the major physiological inhibitor of tissue type plasminogen activator. PAI-1 is an inhibitor of fibrinolysis and increase in its levels are considered to contribute to the pro-coagulant state associated with thrombotic diseases.

In the past study it was found that PAI-1 level decreased in both intensive insulin therapy and /or combined with metformin (15). Consistent with these results it has been reported that glucose control and insulin treatment improved fibrinolysis by reducing PAI-1 concentration/ activity (16) and impaired fibrinolysis during sepsis has been associated with widespread activation of coagulation through the release of inflammatory cytokines (17).

Nitrotyrosine was significantly lower in the EPO treated patients at the days of 3, 6 and 9 after treatment compared with control patients; in addition its level decreased significantly at the days of 3 and 6 in the EPO group in comparison to their baseline values.

EPO may exert its anti oxidative effects directly by exploiting intracellular anti oxidative mechanisms. Increase in the red blood cells by EPO may also indirectly reduce cellular oxidative stress, as red blood cells are loaded with a substantial amount of antioxidative enzymes.

A comparison of SOFA scores on the first, third and seventh day showed that EPO group had lower SOFA score which at least indicate that EPO has anti-inflammatory activity.

SOFA score was selected as a quantitative tool to judge this matter to some extent because it represents the dynamics of illness including the effectiveness of therapy.

Among critically ill patients, erythropoietin uses appears to be associated with increased thrombotic events. This is consistent with the results of recent

trials involving non-critically ill patients with either cancer or chronic renal failure. In these studies, when erythropoietin was used to achieve higher target hemoglobin concentrations (i.e., > 120 g/l), the risk of thrombotic complications and death were increased (18, 19). Importantly, patients with past thromboembolic complications had to be strictly excluded, and therefore patients who are prone to bleed should not be included in a chronic treatment study. However findings of this study did not show any thromboembolic complications because Platelet counts and other related factors had been carefully and continuously monitored at all times of EPO treatment and even in weeks thereafter. Other side-effects such as hypertension and allergic reactions

have been reported (20).

CONCLUSION

Administration of recombinant human erythropoietin for ICU-admitted traumatized patients seems beneficial as it may help to restrain the inflammation and oxidative injuries, which in turn may lead to improved prognosis during and after ICU admission.

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REFERENCES

1. Murray CJL, Lopez AD. The global burden of disease: a comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020. Cambridge, Mass: Harvard University Press; 1996.
2. Corwin HL, Gettinger A, Pearl RG, Fink MP, Levy MM, Abraham E, MacIntyre NR, Shabot MM, Duh MS, Shapiro MJ. The CRIT Study: Anemia and blood transfusion in the critically ill--current clinical practice in the United States. *Crit Care Med*, 2004; 32:32-59.
3. Najafi A, Mojtahedzadeh M, Mahmoodpoor A, Aghamohammadi M, Ahmadi A, Nahreini S, Pazuki M, Khajavi MR, Abdollahi M. Effect of N-acetylcysteine on microalbuminuria in patients with acute respiratory distress syndrome. *Arch Med Sci*, 2009; 5:408-414.
4. Darlow BA, Buss H, McGill F, Fletcher L, Graham P, Winterbourn CC. Vitamin C supplementation in very preterm infants: a randomized controlled trial. *Arch Dis Child Fetal Neonatal Ed*, 2005; 90:117-122.
5. rion LP, Bell EF, Raghuvver TS. Vitamin E supplementation for prevention of morbidity and mortality in preterm infants. *Cochrane Database Syst Rev*, 2008; 149:9-15.
6. Mousavi, S., Mojtahedzadeh, M., Abdollahi, M. Place of iron chelators like desferrioxamine and deferasirox in management of hyperoxia-induced lung injury; a systematic review. 2010; 6:397-408.
7. P Katavetin, K Tungsanga, S Eiam-Ong and M Nangaku. Anti oxidative effects of erythropoietin. *Kidney Int*, 2007; 72:10-15.
8. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. *Crit Care Med*, 1985; 13:818-829.
9. Baker SP, O'Neill B, Haddon W, Long WB. The Injury Severity Score: a method for describing patients with multiple injuries and evaluating emergency care. *J Trauma*, 1974; 14:187-196.
10. Vincent JL, de Mendonca A, Cantraine F, Moreno R, Takala J, Suter PM, Sprung CL, Colardyn F, Blecher S. Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicenter, prospective study. *Crit Care Med*, 1998; 26:793-1800.
11. Marsden JT. Erythropoietin-measurement and clinical applications. *Ann Clin Biochem*, 2006; 43:97-104.
12. Soltan-Sharifi MS, Mojtahedzadeh M, Najafi A, Khajavi MR, Rouini MR, Moradi M, Mohammadirad A, Abdollahi M. Improvement by N-acetylcysteine of acute respiratory distress syndrome through increasing intracellular glutathione, and extracellular thiol molecules and anti-oxidant power: Evidence for underlying toxicological mechanisms. *Human and Experimental Toxicology*, 2007; 26:697-703.
13. Zhang J, Li Y, Cui Y, Chen J, Lu M, Elias SB, Chopp M. Erythropoietin treatment improves neurological functional recovery in EAE mice. *Brain Res*, 2005; 1034:34-39.
14. Agnello, D., Bigini, P., Villa, P., Mennini, T., Cerami, A., Brines, M.L., Ghezzi, P. Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. *Brain Res*, 2002; 952:128-134.
15. Mojtahedzadeh M, Rouini MR, Kajbaf F, Najafi A, Ansari G, Gholipour A, Mofid AR, Abdollahi M. Advantage of adjunct metformin and insulin therapy in the management of glycemia in critically ill patients. Evidence for nonoccurrence of lactic acidosis and needing to parenteral metformin. *Arch Med Sci*, 2008; 4:74-81.
16. Monica Savioli; Massimo Cugno; Federico Polli; Paolo Taccone, Giacomo Bellani, Paolo Spanu; Antonio Pesenti, Gaetano Iapichino, Luciano Gattinoni, FRCP. Tight glycemic control may favor fibrinolysis in patients with sepsis. *Crit Care Med*, 2009; 37:424-431.
17. Hamishehkar H., Beigmohammadi M.T., Abdollahi M., Ahmadi A., Mahmoodpour A., Mirjalili M.R.,

- Abrishami R., Khoshayand M.R., Eslami K., Kanani M., Baeeri M., Mojtahedzadeh M. Identification of enhanced cytokine generation following sepsis. Dream of magic bullet for mortality prediction and therapeutic evaluation. *Daru*, 2010; 18:155-162.
18. Henke M, Laszig R, Rube C, et al. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomized, double-blind, placebo controlled trial. *Lancet*, 2003; 362:1255-1260.
 20. Singh AK, Szczech L, Tang KL, Barnhart H, Sapp S, Wolfson M, Reddan D. Correction of anemia with epoetin alpha in chronic kidney disease. *N Engl J Med*, 2006; 355:2085-2098.
 21. Locatelli F, Olivares J, Walker R, et al. Novel erythropoiesis stimulating protein for treatment of anaemia in chronic renal insufficiency. *Kidney Int*, 2001; 60:741-747.