Impact of monocytic cytokines in polytrauma patients with orthopedics injuries

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ABSTRACT

Objective: Orthopedic injuries are a growing epidemic affecting predominantly, the young population, after trauma. Polytrauma patients with a femoral fracture and with Injury Severity Score of >15 are of special concern because of complications like Systemic inflammatory response syndrome (SIRS), Multi-organ dysfunction syndrome (MODS) and sepsis. Against this background. We aimed to assess the role of monocytic cytokines in the development of complications in patients, having isolated diaphyseal fracture of femur as compared to those having diaphyseal fracture of femur along with ISS score >15.

Methodology: Patients were divided into to two groups: in first group, only those patients who had isolated femur fracture were included (named as 'Group A'). In the second groups patients having femur fracture along with ISS >15 at the time of admission (named as 'Group B'), were included. The study used flowcytometry based intracellular cytokine assay to circumvent the problem associated with extracellular cytokine assay.

Results and Conclusion: A total of 20 patients aged between 20 and 55 years, presenting to the emergency department within 24 h of injury were enrolled in Group 'A' and 'B' as per criteria mentioned above. Intracellular expression of cytokines in isolated femur fracture tends to normalize towards healthy control in the late phase of trauma. Elevated levels of IL-8 and IL-6 levels in late phase (Day 10) of trauma. IL-8 and IL-6 may increases to compensate the higher levels of IL-1 β . The effect of cytokines on the severity of injury was observed. This complex action of immune cells and proinflammatory cytokines were seen in initial and later stage of trauma.

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1. Introduction

Orthopedic injuries are a growing epidemic affecting predominantly young population after trauma. Fractures of femur are common and are often associated with prolonged immobilization and morbidity in young population.¹ Femoral shaft fractures are characterized by major blood loss and soft tissue damage and could contribute to early inflammatory response.² Polytrauma patients with a femoral fracture and with Injury Severity Score of >15 are of special concern because such patients are also prone to develop

complications like Systemic inflammatory response syndrome (SIRS), Multi-organ dysfunction syndrome (MODS) and sepsis, especially if they survive the initial resuscitation period after injury.^{3,4}

The production of pro inflammatory cytokines like TNF- α , IL- 6, IL-8, and IL- 1 β may predispose to post trauma complications immediately after injury.⁵ These immunological disturbances are the main drivers for post-trauma morbidity and mortality.^{6–8} Monocytes have been suggested to be one of the major sources of pro-inflammatory mediators in the early post-traumatic course and might contribute to infectious susceptibility and late mortality in critical illness.⁹ With the increasing population of young adults being admitted with severe trauma, studies are urgently needed to develop and evaluate diagnostic and prognostic markers of SIRS, sepsis and MODS and to develop treatment strategies for them.

Against this background and considering the implication of

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blood loss and soft tissue injury in long bone fractures, we intended to focus on the imbalance of monocytic cytokines associated with blood loss and the timing of surgical procedure after orthopedics injury. In this study, we aimed to assess the role of monocytic cytokine activity in development of complications in patients having isolated diaphyseal fracture of femur as compared to those having diaphyseal fracture of femur along with ISS score >15. The study used flowcytometry based intracellular cytokine assay to circumvent the problem association with extracellular cytokine assay.

2. Methodology

Fresh trauma cases presenting to the JPNATC of AIIMS with polytrauma & long bone were included in the study, after initial diagnosis in causality department.

Inclusion and exclusion criteria: Patients between the age of 16–65 yrs, having femur fracture or polytrauma with femur fracture who were admitted to our hospital setting within 12 h of injury were included. Patient who had history of intake of antibiotics, steroids or any medication a week before the admission were excluded.

Group distribution: Patients were divided into two groups: in first group, only those patients who had isolated femur fracture were included (named as 'Group A'). In the second groups patients having femur fracture along with ISS >15 at the time of admission, named as 'Group B', were included. Detailed clinical documentation of enrolled patients at the time of admission and during the hospital stay was done to correlate the results with the clinical outcome of the patients. Type and number of surgical procedure was recorded to assess the role of surgery as a second hit on the patient's immune system.

Collection of blood and stimulation: Five ml of whole blood were collected from each patient and divided equally in heparinized (BD vacutainer lithium heparin 75 USP units, BD frankline lakes NJ, USA) and plain vials from all patients on the day of admission in emergency department and on days 2, 5 and 10 thereafter as follow-ups to look for the patient's clinical outcome and correlate it with monocytic activity. The blood was processed immediately for Intracellular cytokine assay to prevent cell death over the period of time. Same amount of blood was taken from healthy controls. The whole blood was challenged with 1 µg/mL lipopolysaccharide (LPS) (*Escherichia coli*, Serotype 055:B5, Sigma-Aldrich, St Louis, MO, USA), incubated at 37 °C in 5% carbon dioxide (CO₂) with 1 µg/mL monensin (BD, San Jose, CA, USA) to inhibit protein secretion for 4 h. In parallel, the other tube, taken as basal (unstimulated) tube was also treated similarly, but without LPS to serve as baseline comparison.

Surface and Intracellular staining of cells for flowcytometric

analysis: After stimulation, blood was incubated with specific monoclonal antibodies for CD 45 and CD14 antigen tagged with PerCP Cy5.5 and PE- Cyanine 7 (BD, San Jose, CA, USA) respectively. After incubation, RBCs were lysed with lysis buffer (BD, San Jose, CA, USA). The cells were then fixed and permeabilized by fixation and permeabilization buffer (BD, San Diego, CA, USA), followed by incubation on ice in dark with intracellular specific antibodies for TNF- α , IL-6, IL8 and IL-1 β tagged with Phycoerythrin (PE) (BD, San Diego, CA, USA), Allophycocyanin (APC) (BD, San Jose, CA, USA) and Fluorescein isothiocyanate (FITC) (BD, San Diego, CA, USA) respectively. The cells were then washed twice with wash buffer (BD, San Diego, CA, USA) and the pellet was resuspended in 1% para-formaldehyde (Fischer-Scientific, Mumbai, India). The cells were then analyzed by flowcytometry.

Multiparameter flow cytometric analysis: Samples were analyzed using multiparametric flowcytometer (BD FACS AriaIII, USA). Results were analyzed using BD FACS Diva software and gate was set around the monocyte population, which was strongly positive for CD14 on forward scatter on dot plots. TNF- α , IL-1 β , IL-6, and IL-8 production from monocytes were then analyzed.

All the patients enrolled in the study were clinically followed-up till their final hospital outcome (discharge/death) and their clinical and laboratory finding were recorded daily including the microbiological culture reports of all samples received in the laboratory. Details of surgical procedure performed and antimicrobial treatment were similarly noted.

Statistics: Results were recorded as mean $\pm$ standard error of the mean (SEM). Data were analyzed by the Mann-Whitney Test for multiple comparisons. Differences were considered significant with P values less than 0.05.

The study was approved by the Institute's ethical committee.

3. Results

3.1. Demographic profile

A total of 20 patients aged between 20 and 55 years, presenting to the emergency department within 24 h of injury were enrolled in Group 'A' and 'B' as per criteria mentioned above. Patients from Group A, isolated femur fractures, were directly admitted to orthopedics unit of hospital, Group B patients, polytrauma with ISS were >15, were admitted in ICU after initial treatment from emergency. Out of the 20 enrolled patients, 9 (45%) had isolated femur fracture and 11 (55%) had polytrauma with long bone femur fracture. Among these 11 polytrauma patients, 3 patients had abdominal injuries, 2 had chest injuries, 2 had both chest and abdominal and 2 had maxiofacial injury along with long bone femur fracture. The detailed demographic profiles of the patients enrolled is given in Table 1.

Table 1
Details of the enrolled in the study.

Total Number of Patients (n = 20)	Age (Mean $\pm$ SED)	ISS (Mean $\pm$ SED)	Common Surgery	Complications	Blood transfusion	Outcome	LOS (average)
Diaphyseal fracture of femur (n=11)	36.6 $\pm$ 12.3	8 $\pm$ 13.5	Open Reduction Internal Fixation (ORIF)	NA	Not required	Discharged	15
Diaphyseal fracture of femur along with ISS >20 (n=9)	31.6 $\pm$ 13	27 $\pm$ 13.6	Open and closed reduction of Internal and external fixation, laparotomy	Organism found in BAL and wound <i>Acinetobacter</i> <i>baumannii</i> <i>Klebsiella</i> <i>pneumoniae</i> <i>Pseudomonas</i> <i>aeruginosa</i>	3 units of blood (Average)	Discharged	19

3.2. Surgical procedure and blood transfusion

Six patients (30%) had underwent open reduction and internal fixation (ORIF) with intramedullary nailing (IMN) of femur whereas 3 (15%) patients had to undergo closed reduction and internal fixation (CRIF), 3 (15%) underwent exploratory laparotomy and 2 (10%) underwent fasciotomy. Of the remaining, 6 (30%) was managed conservatively hence no surgery was required for these patients. ORIF with IMN and CRIF were planned after 48 h of admission of the patients whereas exploratory laparotomy and fasciotomy was done immediately after admission. Blood transfusion was required for 3 patients in group 'B' during surgery. Out of these three, two had severe abdominal trauma along with femur fracture, underwent ORIF and exploratory laparotomy and require 3 units of RBC. Another patient had both lower leg proximal tibial fracture with mild chest and abdominal injuries and underwent grafting of left popliteal artery and external fixation of left tibia and required 4 units of RBC.

All the patients were discharged after a variable length of hospital stay. Average length of hospital stay for group A and group B were 15 and 19 days respectively. None of the patients developed post trauma complications like MODS, SIRS or sepsis.

During the hospital stay, only two patients developed infection. One of them developed VAP in ICU due to *Acinetobacter baumannii*, *Klebsiella pneumonia* and one had a wound infection due to *Pseudomonas aeruginosa* in BAL.

3.3. Intracellular cytokines levels in two groups of orthopedics trauma patients

3.3.1. TNF- α

Levels of TNF- α in both the groups were significantly decreased on day 0, **CD14+TNF- α , 53 ± 8 ($p = 0.00743^{**}$)** for Group A; **CD14+TNF- α , 31 ± 9 ($p = 0.0942$)** for Group B, as compared to healthy control, **CD14+TNF- α , 66 ± 3** . The levels reached to its lowest level on day 2 and increased on day 5 and day 10. In Group B, on day 2 the levels were significantly reduced down, **CD14+TNF- α , 20 ± 9 ($p = 0.0018^{**}$)** and went up in further follow ups. There was no significant difference between the two groups; both the groups followed the same pattern of cytokines levels.

3.3.2. IL-6

Levels of IL-6 increased immediately after trauma **CD14+TNF- α , 24 ± 8** for group A; **CD14+TNF- α , 17 ± 3** for group B as compared to healthy control, **CD14+TNF- α , 17 ± 3** . Levels decreased further on Day 2 and gradually started to increase towards healthy control level from day 5. No significance difference was found between the two groups. However, as compared to isolated femur fracture, higher IL-6 was found in the patients with higher ISS.

3.3.3. IL-1 β

In the early phase of trauma IL-1 β was found to be elevated in both the study groups of orthopedics trauma as compared to healthy control, **CD14+IL-1 β , 8 ± 0.9** . On day 2, decreased IL-1 β level, **CD14+IL-1 β , 8 ± 4** , was found in the patients with isolated femur fracture whereas, the levels were found to be shoot up (**IL-1 β CD14+IL-1 β , 14 ± 9), in patients with ISS>20 along with femur fracture. Lower levels of IL-1 β were seen on day 5 till day 10 in both the groups of patients. Pattern of IL-1 β on Day 2 differed in group B, otherwise the pattern in further follow ups was found to be similar in all the enrolled patients.**

3.3.4. IL-8

Elevated level of IL-8 was found in group B, **CD14+IL-8, 14 ± 6** as compared to Group A, **CD14+IL-8, 7 ± 4** , although as compare to

Table 2
Cytokines levels in the two groups of orthopedics patients.

Time	TNF- α		IL-6		IL-1 β		IL-8	
	Control	Isolated fracture of femur (N=11)	Control	Isolated fracture of femur (N=11)	Control	Isolated fracture of femur (N=11)	Control	Isolated fracture of femur (N=11)
HCs	66 $\pm$ 3							
Day 0		53 $\pm$ 8	17 $\pm$ 3	24 $\pm$ 8	8 $\pm$ 0.9	9 $\pm$ 6 (0.0110*)	6 $\pm$ 1	14 $\pm$ 6
Day 2		31 $\pm$ 9 (p = 0.0073*)		17 $\pm$ 6.3	8 $\pm$ 4	11 $\pm$ 4 14 $\pm$ 9	7 $\pm$ 4 18 $\pm$ 11	17 $\pm$ 10
Day 5		41 $\pm$ 9 (p = 0.0284*)		18 $\pm$ 5	3 $\pm$ 0.8 (p = 0.0051**)	6 $\pm$ 4	8 $\pm$ 8	25 $\pm$ 7
Day 10		50 $\pm$ 5		29 $\pm$ 0.8	5 $\pm$ 2	6 $\pm$ 1	1 $\pm$ 0.7	20 $\pm$ 7 (p = 0.0476*)

*: Statistically significant

HC, **CD14+IL-8, 6 ± 1** , the levels were higher in both the groups of patients. Patients with isolated femur fracture tended to normalize on Day 5 and Day 10. In contrast, multiple trauma patients, enrolled in Group B, the levels were significantly increased till day 10.

Table 2 shows the cytokine levels of TNF- α , IL-6, IL-8 and IL-1 β at different interval. Fig. 1 shows the comparison in levels of various cytokines between the two groups.

4. Discussion

In the present study we focused on the orthopedic injuries which included isolated long bone fracture compared with another group of femur fracture who had ISS >15. Advancement in the surgical and critical care technology and hospital care has reduced the mortality in trauma patients. However, fatalities due to post trauma complication remains a major problem.^{10,11}

To understand the complex action of immune cells in initial and later stage of trauma sophisticated technology and patent follow-ups are needed. In the present study, we focused on the role of monocytic cytokines in isolated femur fracture and compare them with fracture femur along with multiply injured patients having ISS>15. We selected the monocytes as they are the first to attack the antigen and phagocytose the cells and trigger the immune system. Our hypothesis was that the levels of intracellular monocytic cytokines will provide important information on the complexity of immune reaction in these patients. In our study, considering the short life span of the cytokines and the effect of external environment on immune cells in vitro, we processed the blood cells within half an hour of sampling to avoid cell death and to understand the complex network of these cytokines. Most of the studies in the literature have been done on plasma cytokines.^{12–14} The levels of IL-6, TNF- α , IL-8 was found to be elevated in most of the study and remained elevated.¹⁵ However, plasma usually predict the overall sum of the cytokine produced by various immune effectors cells. Also, most studies lack follow ups of patients.

In a study by Hietbrink et al., in 2011, the level of plasma IL-6 was significantly increased in severely injured patients compared to patients with isolated femur fractures and matched controls and showed a lower percentage HLA-DR positive monocytes.¹⁶ The same pattern of plasma cytokine were observed by Husebye et al., in 2012. They could not find a significant difference in the four studied cytokines.¹⁷ Another study by Smith et al. showed a significantly higher IL-10 release and a significant depression in the expression of HLA-DR on monocytes at different time intervals.¹⁸ The main drawbacks of these studies were that, they all used the plasma as sample source. We have targeted monocytes to demonstrate the variation in the level of cytokines produced in different phase of trauma at cellular level.

In our study, a decrease in the levels of TNF- α was found in early trauma (day 0), in both the group. A statistically significant dip was observed in TNF- α on day 2 in both the groups which demonstrates the effect of surgery on the production of TNF- α in these patients. Patients with multiple injuries with femur fracture showed a higher level of cytokines on day 5 and day 10.

The levels of IL-1 β , IL-6 and IL-8 were found to be higher in group B as compared to Group A. IL-1 β levels were decreased in isolated femur fracture patients. On follow ups and tended to normalize; where in group B, on day 2 the level elevated after orthopedic surgery and lowered down slowly in next few days. Similar pattern was seen in group A IL-8 levels.

IL-6 acts as both a pro and anti-inflammatory cytokine during infection and after trauma, which further leads to inflammation. IL-8 is a chemokine and play an important mediator of the immune reaction in the innate immune response. In our study, we found that the IL-6 levels, after a dip on day 2 showed increased levels on further follow ups in both the groups. In contrast, increased level of IL-8 was observed over the followups in the group of critically ill patients only. Our results indicates that IL-8 and IL-6 might predicting an anti-inflammatory effect in the enrolled orthopedics patients.

Fig. 1. Showing the levels of TNF- α , IL-6, IL-1 β and IL-8 and comparing the levels between the two studied groups of orthopedics. **A.** Showed the levels of TNF- α . **B.** Showed the levels of IL-6. **C.** showed the levels of IL-1 β and **D.** showed the levels of IL-8.

We have observed a decreased level of IL-8 and increased level of IL-6 in isolated femur fracture after day 2, which might suggest the compensation of excessive IL-8 by IL-6 in these patients. IL-8 and IL-6 statistically increased whereas other cytokines tended to normalized towards the levels healthy control on day 10. This may suggest anti inflammatory effect of cytokines which compensates the excessive amount of pro inflammatory at the time of injury.

Another aspect which was studied was the impact of timing of surgical procedures on the cytokine levels in the two groups of patients. We found that the effect of orthopedics surgery to fix the femur fracture acts as second hit in critically ill ICU patients. Our study clearly shows that the levels of cytokines were high in more severely ill patients as compared to patients with isolated femur fracture. The levels of TNF- α and IL-6 dipped on day 2 whereas IL-1 β and IL-8 showed higher level on day 2. In the patients with ISS>15 the femur fracture was stabilized by intramedullary nailing after 48 h of admission and these surgeries which acted as second hit to activate the immune cells.

In conclusion, the effect of severity of orthopedic trauma on the cytokines level was studied. Intracellular expression of cytokines in isolated femur fracture tends to normalize towards healthy control in the late phase of trauma. Femur fracture along with ISS>15 showed a variation in the levels of monocytic cytokines. Elevated levels of IL-8 and IL-6 levels in late phase (Day 10) of trauma. Second Timing of the surgery is important, since it acts as a second hit to induce inflammation in multiple trauma patients and affect the monocytic cytokine levels. IL-8 and IL-6 may increases to compensate the higher levels of IL-1 β . There are few studies on the intracellular cytokines in orthopedics trauma; no study with prolonged follow ups has been done to assess the monocytic activity in orthopedics trauma patients. A limitation of our study was the less number of patients and major strength of our study is the prolonged clinical follow ups and the use of flowcytometry based monocytic assay.

Conflict of interest

All authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jcot.2018.08.004>.

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