Original Research Article

Evaluation and comparison of oxidative stress levels in patients with proximal tibial fracture

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Abstract

Background: It was assumed that every tissue and bone injury cause oxidative stress. The primary objectives of the present study were to assess the concentration of total nitric oxide, the unstable forms of nitric acid, malonyldialdehyde indicator for the lipid peroxidation and the endogenous ferric reducing antioxidant power assay (FRAP) continuously monitored over a four-week period in the serum samples of patients with proximal tibial fractures. **Materials and Methods:** The study recruited volunteers as a control group of 20 healthy people, and in another group, 20 patients suffering from proximal tibial fractures were undergoing inpatient and outpatient treatments. The total nitric oxide concentration in the blood serum was determined according to the Griess reaction, while the concentration of malonyldialdehyde, the reactive nitrogen intermediates and Citrulline and the endogenous ferric reducing antioxidant power assay (FRAP) were estimated using the standard biochemical methods. **Results:** Results were analysed with respect to the measurement time. We observed significantly higher concentrations of total nitric oxide and lower concentrations of malonyldialdehyde were observed in the blood serum of patients with proximal tibial fractures compared to the control group. There were no statistically significant differences in the reactive nitrogen intermediates and citrulline levels from the serum of patients throughout the study period. However, a significantly higher endogenous ferric reducing antioxidant power assay (FRAP) concentration was measured in the patient serum sample on the first day of surgery compared with the control group. Oxidant levels rise by the second and third week, perhaps due to callus formation and angiogenesis, which results in reperfusion at the fracture site. Oxidative stress may also be proportional to the extent of tissue injury, and severity of bone fractured. **Conclusions:** Our results revealed that the intensity of local processes resulting from proximal tibial fractures was related to the levels of nitric oxide, confirming its significant role to combat the detrimental effects of oxidative stress. It also acts as an indicator in the healing process of bone fracture. A better understanding of these mechanisms may help in defining the role of oxidative stresses after fracture and perhaps better define the role of antioxidants in helping fracture healing.
Keywords: Proximal Tibial Fracture, Antioxidant, Nitric Oxide; Oxidative Stress Malonyldialdehyde.

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INTRODUCTION

Each tissue injury, even a tiny one, induces a systemic reaction in the form of inflammation. Oxygen-free radicals are considered a major common final pathway of tissue injury in different organ systems.[1] Oxidative stress is the chain of oxidative events that leads to increased production of reactive oxygen species that cause tissue injury. Following a fracture, oxidative stress injury may be caused by an ischemia-reperfusion mechanism.[2] This results in numerous antigens reaching the extracellular space penetrating from the outer environment and inside damaged cells. The macrophages, lymphocytes and damaged endothelial cells in and around the site of injury, theses cells secretes various types of cytokines and proinflammatory factors, these factors activates the immune system to synthesise significant concentrations of nitric oxide (NO) through sustained stimulation of nitric oxide synthase (iNOS).[3,4] The first three days of fracture

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healing may be compared to the ischemia period, where no oxidative stress injury occurs. After this, in the stage of callus formation, in addition to fibroblast and collagen cells, new capillary vessels with other inflammatory cells increase the production of oxygen free radicals. These radicals may cause oxidative injury to the fractured bone, as seen in other tissues with reperfusion injury.[5] Due to the attack of oxygen free radicals on the lipid component of the cell membrane, the lipid peroxide content is elevated. To evaluate this, the estimation of levels of many intermediate lipid peroxides and their end-products has been used to evaluate oxidative stress indirectly. The most reliable indicators are Malonyldialdehyde (MDA) or Thiobarbituric acid-reducing substances (TBARS).[6] The involvement of nitrous oxide (NO) as an effector molecule in many physiological and pathological situations has been established. The involvement of Nitrous oxide (NO) as an effector molecule in many physiological and pathological situations has been firmly established.[7,8] We attempted to study oxidative stress after fractures by evaluating the blood levels of nitric oxide (NO) and malonyldialdehyde (MDA). NO is chemically unstable, and hence its levels were monitored indirectly by measuring the levels of reactive nitrogen intermediates (RNI) and Citrulline (CIT); we also evaluated the endogenous antioxidant status by ferric reducing antioxidant power (FRAP) assay at weekly intervals. These estimations might help understand the relationships between the secretion of NO and its role in the process of bone fracture healing.

MATERIALS AND METHODS

Subject: The control group comprised 20 healthy individuals (men) who voluntarily agreed to participate in this study, aged 20–30 years and had no systemic diseases and bone fractures within the last five years. The patients group consisted of 20 individuals (men) aged 20–37 years, suffering from proximal tibial fractures and undergoing inpatient and outpatient treatments in Kamineni Institute of Medical Sciences, Narketpally. All the participants were thoroughly briefed about the objectives of this study, and written informed consent was obtained from all the participants from both groups.

Blood Sample Collection: Five millilitres of blood were collected by venous arm puncture under aseptic conditions into test tubes with a clot activator. Blood was collected at four-time points: on the day of surgery, at seven days after surgery, at 14 days, at 21 days and 28 days after surgery. The serum sample was obtained by centrifugation of blood samples at 2000rpm for 10min, and it was stored at −80◦C until the date of analysis.

Estimation of Total NO Concentration in Serum: Total NO concentration was determined as the sum of NO_2^- and NO_3^- concentrations in an indirect method based on measurement of NO_2^- concentration in serum according to Griess reaction.[9,10] Nitrite concentrations were measured with the help of a spectrophotometer at 540 nm. The calorimetric method of Boyde and Rahmatullah was followed for the estimation of Citrulline using diacetyl monoxime,[11] MDA levels were estimated by the Ohkawa H *et al.*[6] method, and the measurement of total antioxidant activity was carried out by the FRAP assay.[12,13]

Statistical Analysis: One-way analysis of variance (ANOVA) was used to determine the statistical significance among groups and the paired "t" test to detect a significant difference between study groups. Statistical significance was set at a level of $p < 0.05$

RESULTS

The control group comprised 20 healthy individuals (men) who voluntarily agreed to participate in this study, these volunteers were aged between 20–30 years, and they had no history of any systemic diseases and bone fractures within the last five years. The patients group consisted of 20 individuals (men) aged 20–37 years, suffering from proximal tibial fractures.

Table 1: Estimation of Nitric Oxide (NO) levels (μM) in the control and patient groups during recovery.

Variables	Control (n=20)	Patients (n=20)
Day 1	49.27 ± 11.29	92.31 ± 22.57*
Day 7		101.57 ± 31.55
Day 14		127.29 ± 42.23
Day 21		79.48 ± 19.36*
Day 28		52.28 ± 9.25

Data represent by Mean ± SE, *p < 0.05, significant **p < 0.01, very significant.

The concentration of total NO was higher in the serum of all the patients with fractures before and after the procedure (Table 1). The statistically significant differences were observed in the total NO concentrations in the serum between the patients with the control group at $p < 0.05$.

Table 2: Estimation of Reactive Nitrogen Intermediates (RNI) levels ((nmol/ml) in the control and patients groups during the recovery period.

Variables	Control (n=20)	Patients (n=20)
Day 1	369.24 ± 28.47	228.21 ± 24.49*
Day 7		273.59 ± 31.28
Day 14		557.28 ± 41.27**
Day 21		339.48 ± 29.24*
Day 28		261.86 ± 19.87

Data represent by Mean ± SE, *p < 0.05, significant **p < 0.01, very significant.

There were a significant rise in the Reactive Nitrogen Intermediates **(**RNI) levels during the 7th day and a peak during the 14th day in the patients group compared to the control group (Table 2). The increase in the patients group was significantly more than the control group. Inpatient group RNI levels were decreased almost to the initial level as on day one by the 28th day.

Table 3: Estimation of Citrulline (CIT) levels ((nmol/ml) in the control and patient groups during the recovery period.

Variables	Control (n=20)	Patients (n=20)
Day 1	1321.42 ± 71.29	1359.34 ± 97.52**
Day 7		1619.81 ± 109.34
Day 14		1931.53 ± 124.82*
Day 21		1249.43 ± 84.31*
Day 28		923.24 ± 54.22

Data represent by Mean ± SE, Significant *p < 0.05, **p < 0.01.

There was a significant elevation in the Citrulline (CIT) levels during the 7th day and a peak during the 14th day in the patients group compared to the control groups (Table 3). These levels were decreased almost to the initial values by day 21.

Table 4: Estimation of Malonyldialdehyde (MDA) levels (µmol/ml) in the control and patient groups during the recovery period.

Variables	Control (n=20)	Patients (n=20)
Day 1	0.064 ± 0.0012	0.52 ± 0.17*
Day 7		0.58 ± 0.12
Day 14		1.23 ± 0.24*
Day 21		0.48 ± 0.08*
Day 28		0.09 ± 0.02

Data represent by Mean ± SE, Significant *p < 0.05, **p < 0.01.

There were a significant increase in the Malonyldialdehyde **(**MDA) levels by the 14th day in the patients group (Table 4). By the 21st and 28th days, MDA levels decreased to less than values seen on day 1.

Table 5: Estimation of Ferric Reducing Anti-Oxidant Power Assay (FRAP) levels (µmol/ml) in the control and patient groups during the recovery period.

Variables	Control (n=20)	Patients (n=20)
Day 1	512.24 ± 61.34	567.92 ± 72.29**
Day 7		592.72 ± 59.27*
Day 14		827.49 ± 81.27*
Day 21		483.51 ± 39.27*
Day 28		298.48 ± 29.84

Data represent by Mean ± SE, Significant *p < 0.05, **p < 0.01.

There were a significant rise in the Ferric Reducing Anti-Oxidant Power Assay **(**FRAP) levels by the 14th day in the patients group compared to the controls (Table 5). By the 28th day, FRAP values again decreased to a level less than those on day 1.

DISCUSSION

In normal healthy bone and muscles, tissues have a significant amount of active eNOS, producing low NO amounts. The local synthesis of this molecule is sufficient to stimulate both these cell types. However, during inflammation, due to the action of inter-alia, proinflammatory cytokines, the activity of iNOS may be restored along with an intensified synthesis of Nitric Oxide (NO). Elevated NO levels in the intracellular fluid and intercellular space may lead to cell apoptosis, including that of pro-osteoclasts and leukocyte deficiency, which contributes to an inhibited alteration of the cytoskeleton.[1-3] Therefore, high concentrations of total NO were observed in the serum of patients with proximal tibial fractures

throughout the study period.[4,5] Radiation, pollution, smoking, alcohol addiction and obesity have adverse effects on the biological systems, and the literature [1-6] has supported some benefits of antioxidants to counter these adverse effects. So much is talked about antioxidants and their role in physiological and pathological conditions that it made us think of their importance in our subset of patients who sustained fractures. This study was designed to evaluate oxidative stress after sustaining a fracture. Corbett *et al.*[14] have shown that increased activity of eNOS in cortical bone and blood vessels at the beginning stages of fracture (on the first day) as well as an increased stimulation of iNOS in the recovery period (2 weeks after injury) in a rabbit model. A similar type of measurement was also reported by Keskin *et al.*,[15] who observed higher concentrations of total NO in the serum of patients with an isolated femur fracture. Prasad *et al.*[16] also demonstrated high NO concentrations in the serum of patients with isolated femur fractures and other fractures in long bones apart from the femur. Gokturk *et al.*[17] evaluated oxidant status during bone healing in rats by using MDA levels in bone specimens to indicate oxidative stress. They noted statistically significant increases in MDA levels on days 7 and 14 after experimentally fracturing the right tibia of the study rats. They concluded that oxidative stress occurs during the second and third weeks after a fracture. Since the MDA levels were evaluated in the bone specimens, they also postulated that this oxidative stress could potentially cause impaired fracture healing.[18-20] The increased values of RNI, cit and MDA by the 7th day, peaking by the 14th day, and the decline over the next two weeks showed a significant level of oxidative stress during the second- and third-weeks post-fracture. The FRAP values, representing the total endogenous antioxidant status, also showed a similar pattern: i.e., rise during the 7th and 14th days and gradual decline by the end of the fourth week. Although we have no clear explanation for this, we presume that it may attempt to combat the rise in oxidant levels. The effect of antioxidants needs to be seen by fixing the timing of surgery. It is also recommended that fracture cases be studied for a more extended period, perhaps until the union is achieved, to see further variations.

CONCLUSIONS

The duration of fracture healing observed in patients with a proximal tibial fracture at different intervals was found to be related with an elevated NO concentration, as well as changes in the concentrations of MDA, indicating that the intensity of local processes is reflected by the changes in the action of NO in the serum. The observations from the present study suggest the applicability of NO's determination in assessing proximal tibial fracture healing. This preliminary study has attempted to create a platform for further research. We assume that the observations of this study may help in understanding fracture healing better.

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