Evaluation of oxidative stress after fractures. A preliminary study

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In a preliminary attempt to see if oxidative stress occurs after major fractures, we evaluated two groups of patients sustaining a single fracture (Group A) and multiple fractures (Group B), and compared them with healthy controls (Group C). Indirect evaluation using plasma was done, as serial samples directly from bone could not be taken in humans. We measured plasma levels of malonyldialdehyde (MDA), which depicts the lipid peroxide content, and the unstable nitric Acid (indirectly through measuring reactive nitrogen intermediates and citrulline), serially over a four-week period. The endogenous ferric reducing anti-oxidant power assay (FRAP) was also done. All these have been proven to be representative of oxidative stress in other situations. We noted significant changes in these values, peaking by the 2nd and 3rd weeks post fracture, and coming down by the 4th week. This implies that oxidative stress does occur after a fracture ; the initial few days are eventless, probably because the fracture causes a local region of ischaemia. Oxidant levels rise by the 2nd and 3rd week, perhaps due to callus formation and angiogenesis, which results in reperfusion at the fracture site. Oxidative stress may also be proportional to the number of bones fractured, as Group B, with multiple fractures had more elevated values. The antioxidant levels also behave similarly to combat the detrimental effect. The pro and antioxidants values then gradually decline by the 4th week, probably because by then the bone starts to organize. 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.

INTRODUCTION

In the last twenty years, more and more emphasis has been laid on the oxygen free radicals as a major common final pathway of tissue injury in different organ systems. Oxidative stress is referred to as the chain of oxidative events that leads to increased production of reactive oxygen species which cause tissue injury. Following a fracture, oxidative stress injury may be caused by an ischemia-reperfusion mechanism (14, 17, 20). The first three days of fracture 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

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radicals. These radicals may cause oxidative injury to the fractured bone as has been seen in other tissues with reperfusion injury (8). As the understanding of oxygen free radical damage in different organ systems is evolving, it is perhaps justifiable to attempt to define the nature of oxidative stress in bone trauma and to evaluate if there are any deleterious effects on fracture healing (8, 12, 24).

Due to the attack of oxygen free radicals on the lipid component of membrane, the lipid peroxide content is elevated. To evaluate this, estimation of levels of many intermediate lipid peroxides and their end-products has been used to indirectly evaluate the oxidative stress. The most reliable indicators are Malonyldialdehyde (MDA) or Thiobarbituric acid reducing substances (TBARS) (8, 12, 24). The involvement of Nitrous oxide (NO) as an effector molecule in many physiological and pathological situations has been firmly established. Most of the previous work relating to oxidative stress after a fracture has been based in the experimental setting (12, 24) with minimum data in humans (2). Since direct bone samples cannot be taken serially in humans, we attempted to study the 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 anti-oxidant status by ferric reducing anti-oxidant power (FRAP) assay at weekly intervals.

PATIENTS AND METHODS

Sixty patients between the ages 18-60 years were taken up for this study. All cases had purely musculoskeletal injuries. The cases were divided into two groups of 30 patients each, with Group A having patients with an isolated femur fracture, and Group B consisting of patients with a femur fracture associated with another long bone fracture. Twenty human volunteers (Group C) with no ailment served as controls, and had the tests done in order to form a standard of comparison.

Five milliliters of heparinised blood was drawn from each patient and evaluated for reactive nitrogen intermediates(RNI), citrulline (cit), malonyldialdehyde (MDA) and total endogenous anti-oxidant activity. Blood samples were taken on days 1, 7, 14, 21 and 28 post fractures.

Estimation of NO as RNI was based on the principle that nitrite in the sample reacts with Griess reagent to form a purple ozo dye which was measured spectrophotometrically by Green's method (8, 9). The estimation of Citrulline was determined calorimetrically using diacetyl monoxime by the Boyde and Rahmatullah method (5), estimation of MDA was done by the Stocks and Dormandy method (22) and the measurement of total anti-oxidant activity was carried out by the FRAP assay (4, 7).

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

In the 60 patients evaluated after trauma, sex distribution was similar (*Group A* -27M/3F and *Group B* -28M/2F), reflecting the disparity of involvement in road accidents between men and women. The control group comprised of 10 males and 10 females. The mean ages in groups A, B and controls (C) were 30.07, 33.77 and 32.85 years respectively. There was no statistically significant correlation between the oxidative stress and either sex or age.

There was a significant rise in the RNI levels during the 7th day and a peak during the 14th day in both groups A and B patients as compared to the controls (table I). The increase in group B was significantly more as compared to group A. In group A these values decreased almost to the initial values as on day 1 by the 28th day. In group B, the values decreased to a level less than those as on day 1.

There was a significant rise in the Citrulline levels during the 7th day and a peak during the 14th day in both groups A and B patients as compared to the controls (table II). The increase in group B was again significantly more as compared to group A. These values decreased almost to the initial values by day 21. On the 28th day, these values also decreased to a level less than those noted on day 1.

Table I — RNI values in the control (C) and study groups (A & B) on days 1, 7, 14, 21, 28 after injury

	Day 1 (mean ± SE)	Day 7 (mean ± SE)	Day 14 (mean ± SE)	Day 21 (mean ± SE)	Day 28 (mean ± SE)
Control (nmol/ml) (n = 20)	358.70 ± 30.50				
A (nmol/ml) (n = 20)	230.39 ± 21.10	282.19 ± 27.34*	540.11 ± 38.89**	344.83 ± 28.32	226.15 ± 22.80
B (nmol/ml) (n = 20)	313.51 ± 41.08	434.76 ± 43.33*	791.95 ± 81.27**	367.63 ± 31.88	237.16 ± 18.60

*$p < 0.05$, significant **$p < 0.01$, very significant.

Table II. — CIT values in the control (C) and study groups (A & B) on days 1, 7, 14, 21, 28 after injury

	Day 1 (mean ± SE)	Day 7 (mean ± SE)	Day 14 (mean ± SE)	Day 21 (mean ± SE)	Day 28 (mean ± SE)
Control (nmol/ml) (n = 20)	1251.94 ± 68.35				
A (nmol/ml) (n = 30)	1261.91 ± 113.38	1583.43 ± 136.14*	1889.34 ± 111.24**	1182.39 ± 65.16	853.61 ± 57.65
B (nmol/ml) (n = 30)	1259.15 ± 40.08	1396.25 ± 56.20*	2088.97 ± 104.72**	1295.13 ± 56.13	983.60 ± 34.06

*$p < 0.05$, significant, **$p < 0.01$, very significant.

Table III. — MDA values in the control (C) and study groups (A & B) on days 1, 7, 14, 21, 28 after injury

	Day 1 (mean ± SE)	Day 7 (mean ± SE)	Day 14 (mean ± SE)	Day 21 (mean ± SE)	Day 28 (mean ± SE)
Control (μmol/ml) (n = 20)	0.0063 ± 0.0013				
A (μmol/ml) (n = 30)	0.47 ± 0.14	0.42 ± 0.13	1.17 ± 0.20**	0.34 ± 0.11	0.08 ± 0.04
B (μmol/ml) (n = 30)	0.60 ± 0.12	0.62 ± 0.14	1.90 ± 0.34**	0.66 ± 0.17	0.56 ± 0.12 **

$p < 0.01$, very significant.

There was a significant rise in the MDA levels by the 14th day in group A patients (table III). In group B, there was slight increase in values on day 7 and a significant rise in values on day 14. The increase in group B was again significantly more as compared to group A. By the 21st and 28th days, MDA levels decreased to less than values seen on day 1.

There was a significant rise in the FRAP levels by the 14th day in both groups A and B patients as compared to the controls (table IV), with a significantly more increase in group B levels. By the 28th day, FRAP values again decreased to a level less than those as on day 1.

DISCUSSION

Growing evidence strongly suggests that antioxidants have the power to prevent diseases such as cancer (19), coronary artery disease (1) and cataract (6). Antioxidant vitamins may improve immune system function and may even delay some of the effects of aging (3, 16). This is probably due to their ability to intercept and extinguish free radicals. They also have some protective role in diabetes (6, 16), Alzheimer's disease (3, 21) and Parkinsonism. Radiation, pollution, smoking, alcohol addiction and obesity have adverse effects on the biological systems and the literature (6, 16) has supported some benefit 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 the importance of these in our subset of patients who sustained fractures. This study was designed in an effort to evaluate oxidative stress that occurs after sustaining a fracture.

Table IV. — FRAP values in the control (C) and study groups (A & B) on days 1, 7, 14, 21, 28 after injury

	Day 1 (mean ± SE)	Day 7 (mean ± SE)	Day 14 (mean ± SE)	Day 21 (mean ± SE)	Day 28 (mean ± SE)
Control (µmol/L) (n = 20)	441.97 ± 59.64				
A (µmol/L) (n = 30)	467.10 ± 78.18	459.62 ± 68.27	773.50 ± 77.56**	478.90 ± 44.74	380.02 ± 40. 48
B (µmol/L) (n = 30)	456.95 ± 70.29	518.07 ± 70.29	948.09 ± 148.05**	532.03 ± 77.34	318.07 ± 49.54

**$p < 0.01$, very significant.

Various studies in the past have linked tissue damage caused by oxygen free radicals to ischaemia-reperfusion mechanism (14, 17, 20). Motivated by these, occasional attempts have been made to study the role of oxidants when a fracture is sustained. Gokturk *et al* (12) evaluated oxidant status during bone healing in rats, by using MDA levels in bone specimens as an indicator of oxidative stress. Statistically significant increases in MDA levels were noted by them on days 7 and 14 after experimentally fracturing the right tibia of the study rats. They concluded that oxidative stress occurs during the 2nd and 3rd weeks after a fracture. The left tibia was used as the control leg in the sense that it was pinned intramedullarly but not fractured, considering this equivalent to a surgical procedure. It was concluded that only pinning the tibia did not increase the MDA levels. Since the MDA levels were evaluated in the bone specimens, they also postulated that this oxidative stress could be a potential cause of impaired fracture healing.

In another recent study in a human population by Basu *et al* (2), urinary levels of 8-iso-PGF2 α were taken as a bio-marker of oxidative stress. Their aim was to investigate whether oxidative stress had any effect on the bone mass in humans. They used 15-keto-dihydro-PGF2 α, an indicator of inflammatory response, as a control. Their findings established a biochemical link between increased oxidative stress and decreased bone density.

No previous study, to our knowledge, has been carried out in humans to link fractures to oxidative stress. This study was thus done as a preliminary attempt to find any correlation and open avenues for future research. We included only those fracture patients who did not have any external soft tissue injury, and excluded cases with associated injuries like head injury (14, 27), blunt chest trauma (11, 20) and blunt abdomen trauma (10), as these have been implicated in oxidative stress and could have given altered values.

To be able to include an appropriate sized cohort of specimens in a short span of time, we expanded the ages of our patient cohort to include cases ranging from 18 to 60 years of age. Additionally, to avoid any exogenous source of antioxidants, we ensured that no vitamin preparations were prescribed to these patients, as this could have potentially altered the values of the total endogenous antioxidant status.

The ideal evaluation of oxidative stress in fracture healing would be from specimens collected from the fracture site. In human conditions, it is impractical and unjustified to take serial specimens from the fracture site ; we therefore chose to use plasma, which contains the by-products of any reaction initiated due to oxygen free radical induced tissue injury ; to our knowledge, this has never been evaluated previously.

The increased values of RNI, cit and MDA by 7th day, peaking by the 14th day, and the decline over the next two weeks showed a significant level of oxidative stress during the 2nd and 3rd weeks post fracture. The increase was significantly more in group B than A, implying thereby that there is more oxidative stress associated with multiple fractures, which seems to be logical.

The reason for the absence of recordable oxidative stress during the first week could be explained by the fact that at the time of a fracture there is interruption of blood flow to the severed bone and plasma levels may not change initially. This regional ischaemia however leaves viable cells in the peripheral regions of this ischaemic zone, which

could be salvaged if managed adequately. If not, these cells may undergo irreversible injury. This period of ischaemia lasts for about a week. Further during the healing process, callus begins to form and new cells are laid down, which includes new capillary vessels. This leads to increased vascularity in the region. There is also an increase in the inflammatory cells which increases the production of oxygen free radicals. These radicals may then lead to oxidative injury as seen in other tissues (8, 9, 14, 15, 17, 20) with reperfusion. This mechanism of oxidative injury is based on the conclusions of previous studies (9, 14, 15, 20) that have been carried out on other tissues which had suffered oxidative injury. The exact mechanism however needs to be confirmed by taking bone tissue samples directly from the fracture site. These then can be subjected to a detailed histopathological and biochemical analysis. In humans, however, it was not possible to take serial biopsies as it was ethically unjustifiable.

The FRAP values, which represent the total endogenous antioxidant status, also showed a similar pattern : i.e. a rise during the 7th and 14th days and gradual decline by the end of the 4th week. Although we have no clear explanation for this, we presume that it may be an attempt of the body to combat the rise in oxidant levels.

Since surgery requires direct handling of the fracture site and may disturb the healing process, we attempted to correlate the date of surgery with the different parameters evaluated. Most of the cases were operated during the 1st and 2nd weeks post injury. However, we could not correlate the surgical insult to the oxidative stress as the time between injury and surgery varied, and often more than one surgical procedure was done on different days (Group B, multiple fractures).

Increased age (2, 6, 26) has been related to osteopenia and increased oxidative stress. We could not completely evaluate variation in oxidative stress levels with age in the present study due to the study sample being too small ; however our data revealed no statistical correlation of age at injury with the level of recordable oxidative stress.

Considering the whole study, a lot more needs to be studied. More stringent conditions for patient selection need to be applied. Ideally, similar fracture patterns need to be taken up for study, and perhaps operated by a similar mode of fixation by the same surgeon. Factors like blood pressure, heart status, diabetes, smoking, alcohol, body mass index, occupation, etc., which have an influence on the oxidative stress need to be taken into consideration. The timing of surgery needs to be fixed. If possible, representative tissue (from bone) needs to be analyzed for the oxidative stress. The effect of antioxidants needs to be seen. It is also recommended that fracture cases should be studied for a longer period of time, perhaps till union is achieved, to see for further variations.

This preliminary study has attempted to create a platform for further research. We have shown our observations and accepted our shortcomings. We hope that the findings of this study can be capitalised to unmask any hidden fact, which may help in understanding fracture healing better.

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