Role of Common Biochemical Markers for the Assessment of Fracture Union

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Abstract The progress of fracture union requires close monitoring. Whereas, clinical examination and radiographic studies assess the outcome, biochemical markers like serum alkaline phosphatase and urinary hydroxyproline reflect the actual status of bone resorption and bone formation over a short time frame. 36 patients of long bone fracture were randomly allocated for the study. When the patient reported to the Department of Orthopedics after fracture, serum and urinary samples were collected and X-ray of the affected part were taken. Subsequent samples were collected and X-ray taken just after management (either operative or conservative), after 3rd, 5th, 8th and 12th week, respectively after onset of fracture. According to the course of callus formation the patients were divided into two groups that progressed to proper union or mal-union. The levels of serum alkaline phosphatase, urinary

total and free hydroxyproline levels were measured and statistically analysed and compared. A statistically significant positive correlation between total urinary hydroxyproline excretion and serum alkaline phosphatase indicate progress towards satisfactory union. Thus, serial monitoring of biochemical markers of bone turnover can be used as an adjunct to clinical and radiological evidence of fracture healing.

Keywords Fracture union · Biochemical markers · Urinary total hydroxyproline · Urinary free hydroxyproline · Serum alkaline phosphatase

Introduction

Bone tissue is subject to remodeling throughout the lifetime of the individual. The continuous remodeling cycle is actuated by the bone remodeling (BR) units [1]. These units are composed of two distinct components—the process of bone resorption by osteoclasts followed by bone formation by osteoblasts. Through the first 10 years of human growth, BR is accelerated and formation exceeds resorption. Both process are in balance during the second to fourth decade of life, attaining a peak bone mass between the age of 30–35 years. From the fifth decade of life, resorption slightly exceeds formation resulting in net negative balance as evidenced by osteoporosis during menopause [2].

BR also plays an integral role in the union of fractures which consists of removal of older bone tissue followed by callus formation. Activation signal stimulate fusion of mononuclear osteoclast precursors to form giant multinucleate osteoclasts. Osteoclasts resorb bone on the skeletal surface forming a lacunae into which acid and enzymes

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are secreted to solubilize and degrade the mineral and protein matrix. Osteoblasts lay down bone matrix which constitutes more than 90% of type I collagen. Mineralization of the matrix with hydroxyapatite forms new bone [2]. Fracture union is thus a proliferate physiological process that requires 3 months for consolidation of long bone fracture, though in many cases it may extend up to 4–5 months time for consolidation of long bone fractures [3]. Close monitoring is essential to assess the progress of the healing during this phase. Clinical examination and radiological assessment are the cornerstone of fracture union. Other approaches for the clinical evaluation of bone status include study of bone mineral density (BMD), Radionucleotide scan, bone histomorphometry and biochemical markers. While X-ray, BMD and radionucleotide scan provide information primarily about the bone macrostructure, integrity, quantity and ultimate outcome of healing [2], only biochemical markers provide a dynamic picture about the underlying process of BR including its turnover, pathogenesis and can differentiate between normal and delayed healing [4]. When searching for markers of BR, molecules of bone and connective tissue origin that may be detected in plasma and/or urine have been targeted [5]. Biochemical markers of bone turnover are generally divided into two subclasses: bone resorption and bone formation markers. The bone resorption markers are related to osteoclast resorption of matrix and include tartarate resistant acid phosphatase and degradation products of type I collagen in protein matrix especially hydroxyproline, telopeptides etc. Bone formation markers are osteocalcin and bone specific alkaline phosphatase produced from osteoblasts [6].

An increase in the alkaline phosphatase activity has been found in the site of callus formation soon after fracture [1]. Increased production of collagen is also associated with an increase in the hydroxyproline which is considered as an index of collagen metabolism [7]. These alterations are reflected in changes in levels of alkaline phosphatase in plasma and total and free hydroxyproline excretion in urine. In this context, an attempt was made in the present study to find out whether any correlation exists between levels of urinary hydroxyproline excretion, serum alkaline phosphatase, with progress of fracture healing to proper union by satisfactory callus formation as assessed from serial radiograph.

Materials and Methods

Total number of 36 (25 M, 11F) adult patients with minimally displaced or undisplaced long bone fractures were included in the study conducted at Medical College,

Kolkata from August 2008 to July 2009. 33 patients suffered from long bone fracture of femur/tibia or humerus while in the other three cases, corrective osteotomy of femur, osteotomy and lengthening for pseudoarthrosis of tibia and deformity correction procedures were followed, respectively. All the fractures were treated within 4–6 days of the injury. Apart from the type of injury, patients were also screened to rule out the presence of any other factor (e.g. bile duct obstruction, pregnancy, growing children) that may contribute to the rise in alkaline phosphatase levels. Patients with normal urea and creatinine levels were included in the study to ensure optimum renal function. Polytrauma patients, patients given calcium supplementation and patients in the extremes of age were excluded from the study. Patients included in the study were asked to avoid diets rich in collagen. Ethical clearance from the Institutional Ethics Committee was duly obtained for the study as also the informed consent was obtained from each patient and control included in the study. 21 healthy subjects served as controls. The mean of the biochemical parameters in the controls served as the base values.

After the onset of management, all the patients were followed up for a period of 12 weeks. Collection of fasting blood (to estimate serum calcium and alkaline phosphatase) and 24 h urinary samples (to estimate urinary creatinine and hydroxyproline) were done as soon as the patients were admitted at Medical College, Kolkata. First sample was taken just after fracture or after osteotomy (in three cases). Average time for definitive management was less than 1 week. Second sample was taken after definitive management either conservative or operative. Following samples were taken at the end of 3rd, 8th, 12th week after the onset of fractures. Total and free urinary hydroxyproline were estimated from the 24 h urinary samples according to the method of Bergman and Loxley, 1970 [8]. Urinary creatinine was estimated by modified Jaffe's method [9]. Fasting blood was collected for estimation of serum alkaline phosphatase by enzymatic method [10] and estimation of serum calcium by O-cresolphthalein complexone method [11]. All reagents used analytical grade reagents bought from SRL.

Serial radiograph of the fracture site was performed in every patient starting with initial one after trauma as also just after management and at 3rd, 8th, 12th week after onset of fracture. All radiographs were analyzed for signs of callus formation, callus mineralization and bone bridging. Serum calcium level was noted to assess the nutritional status and eliminate patients suffering from hypocalcaemia. Biochemical results were analyzed to assess the progression of the underlying process of fracture union through callus formation and to correlate it with the clinical and radiological parameters.

Results

In respect of the biochemical parameters assessed, mean value and standard deviation were calculated in the control and the patient groups and compared between the two groups. *P*-value is calculated to find the level of significance between the control and patient samples. Except for serum calcium and urinary creatinine all other parameters had statistically significant level of difference when compared between patient group (both proper and impaired union) and control group. The serial increase/decrease of the urinary and serum parameters in the groups that progressed to proper union/malunion are summarized in Tables 1 and 2. As evident from Table 3, a positive correlation exists between the urinary total hydroxyproline excretion and serum alkaline phosphatase levels in the 12th week sample in case of patients with adequate callus formation.

Discussion

Clinical examination and radiological assessment is the cornerstone of fracture union. Other approaches for the clinical evaluation of bone status include study of BMD, Radionucleotide scan, invasive techniques like histomorphometry from iliac crest and measurement of biochemical markers. X ray, BMD and radionucleotide scan provide information primarily about the bone macrostructure, integrity, quantity and ultimate outcome of healing. Histomorphometry is limited to a small area of cancellous bone of iliac crest and does not reflect bone turnover in other parts of the skeleton [2]. On the other hand, Biochemical markers provide a dynamic view of the remodeling process, reflecting the rate of turnover and overall net changes of resorption and formation [4]. Furthermore they can be used to monitor short term effects of therapy and provide early indication of any impairment of healing process. Assay procedures of the biochemical markers are inexpensive and can be repeated several times in a single patient [6]. As the process of bone resorption and formation are complementary to each other, the present study focuses on the status of a bone resorption marker—urinary

excretion of free and total hydroxyproline (an index of type I collagen metabolism) and a bone formation marker—alkaline phosphatase (released from osteoblast membrane) in cases of successful and malunion of fractures.

Hydroxyproline is found mainly in collagen, imparts stability to the molecule and represents about 13% of the amino acid content collagen [7]. Hydroxyproline is derived from proline by a post translational hydroxylation occurring within the peptide chain. As free hydroxyproline released during degradation of collagen cannot be reutilized in collagen synthesis, most of the endogenous hydroxyproline present in biological fluids is derived from various forms of collagen [12]. As half of human collagen resides in the bone where its turnover is probably faster than in the soft tissues excretion of hydroxyproline in urine is regarded as a marker of bone resorption [13].

The mean value of urinary free hydroxyproline was 5.6 mg/day (Normal range 3.9–6.6 mg/day in 24 h urine sample) and urinary total hydroxyproline was 7.5 mg/day (normal range 5.7–9.0 mg/day in 24 h urine sample) in the control group. The pretreatment level of free and total urinary hydroxyproline excretion was 8.46 and 10.41 mg/day, respectively in patients with adequate callus formation. Other studies have also documented a two fold rise of urinary excretion of free and total hydroxyproline following fractures as compared to healthy individuals [14]. With gradual increment, the highest level of free hydroxyproline reached 13.88 mg/dl and total hydroxyproline reached 15.39 mg/dl in a mean time of 8 weeks. Thereafter, the levels decreased gradually up to the 12th week as evident from Fig. 1. This trend of gradual elevation up to the 8th week is due to the fact that the actual phase of resorption continues for 50 days, residual elevation thereafter is a result of ongoing collagen synthesis [15]. On the contrary, in cases of malunion there is a decrease in level after the second sample with the initial mean value at 7.57 mg/day for free urinary hydroxyproline and 10.11 mg/day for total urinary hydroxyproline. The ultimate level at the end of 12th week is 1.10 and 1.82 mg/day for free and total urinary hydroxyproline levels. Decreasing levels of hydroxyproline reflect some amount of initial resorption followed by inadequate callus formation [14] (Fig. 2).

Table 1 Levels of common biochemical parameters after fracture and after management

Sl no.	Parametres	Control	After fracture		After management	
			Proper union	Malunion	Proper union	Malunion
1.	Free U HP (mg/day)	5.71	8.46	7.11	10.55	4.26
2.	Total U HP (mg/day)	7.52	10.41	10.26	12.44	6.78
3.	Free U HP:UCr	0.004	0.007	0.006	0.008	0.004
4.	Total U HP:UCr	0.005	0.833	0.008	0.010	0.005
5.	Alkaline Phosphatase (IU/l)	142	319	180	417	185

Table 2 Serial estimation of common biochemical parameters on the 3rd, 8th and 12th week after fracture

Parametres	3rd Week after fracture		8th Week after fracture		12th Week after fracture	
	Proper union	Malunion	Proper union	Malunion	Proper union	Malunion
Free U HP (mg/day)	12.80	2.37	13.88	1.37	12.46	1.11
Total U HP (mg/day)	14.38	3.87	15.39	2.28	13.57	1.18
Free U HP:UCr	0.011	0.003	0.011	0.001	0.010	0.001
Total U HP:UCr	0.114	0.003	0.121	0.002	0.108	0.001
Alkaline phosphatase (IU/l)	528	202	592	183	476	187

Table 3 Comparison of the correlation coefficients between the common biochemical parameters

Parametres	Correlation coefficient	
	Proper union	Malunion
Urinary free HP versus Se Alk. phosphatase	0.2408	0.2379
Se calcium versus Se Alk. phosphatase	0.1729	0.2371
Urinary total HP versus Se Alk. phosphatase	0.4012*	0.1392

* Statistically significant

The skeletal alkaline phosphatase is an enzyme localized in the membrane of the osteoblasts. It is released into the circulation by a mechanism that is not clear. Though, several tissues contain different isoenzymes of alkaline phosphatase, the liver and bone isoenzymes are the major contributors to the serum levels. Serum total alkaline phosphatase activity is the most commonly used marker of bone formation. Experimentally induced fractures demonstrate a significant elevation of serum alkaline phosphatase within 4 h in rats but not until 1 week in dogs [16]. In human subjects serum alkaline phosphatase shows an

Fig. 1 Graphical representation of changes in free urinary hydroxyproline levels during serial visits for cases of fracture with satisfactory healing versus impaired healing **Fig. 2** Graphical representation of total urinary hydroxyproline levels in cases of fracture with satisfactory healing versus impaired healing

increasing trend just after fractures in cases where adequate union is achieved. Figure 3 traces the levels of serum alkaline phosphatase with a minimum initial value of 319 IU/l. At a mean period of 8 weeks a peak value of 592 IU/l is obtained. After the 8th week the serum alkaline phosphatase levels decrease gradually but do not return to normal as a complete turnover cycle in BR requires an average period of 200 days [17]. The persistent elevated levels above the normal value may be explained by the continuing osteoblastic activity. The gradual decrease of serum alkaline phosphatase value at the end of 8th week as evident from Fig. 3, is consistent with findings of Lal and Jacob [18]. It may thus be inferred that values of serum alkaline phosphatase can be used as biochemical marker of fracture healing in the absence of any other systemic disorder provided the values are obtained systematically at regular intervals. However, the estimation of bone specific plasma alkaline phosphatase (BSAP) could be a better prognostic indicator. In cases of impaired union serum alkaline phosphatase levels became static after a mild initial elevation indicating deficient osteoblastic activity.

Statistical tests for correlation among the different parameters assessed revealed that total urinary hydroxyproline level bears a significant positive correlation to the

Fig. 3 Graphical representation of serum alkaline phosphatase levels in cases of fracture with satisfactory healing versus impaired healing

serum alkaline phosphatase (Table 3) in the group of patients achieving adequate union as per the 12th week sample. Thus, the statistically calculated correlation coefficient of above two parameters can be used to monitor the progress of bone repair.

However, previous studies [19] have established that, the ratio of urinary hydroxyproline to urinary creatinine excretion in a 24 h collection of urine is an improvement on the hydroxyproline excretion alone because it gives more consistent results for subjects of the same age. Subsequently, it has been shown that the ratio determined in random samples of urine is similar to that found in a 24 h collection [20].

Thus, the ratio of urinary hydroxyproline to urinary creatinine was also included as a parameter in the present study. The urinary hydroxyproline creatinine ratio gradually increased during healing process of bones (Table 1). The maximum values were seen around 8 week in those cases which progressed to unite satisfactorily (Table 2). Results of ratio of urinary hydroxyproline and creatinine showed the same trend as of 24 h urinary hydroxyproline excretion, hence this ratio can be used for assessing random samples.

Conclusion

Thus, it may be concluded that serial monitoring of biochemical markers of bone turnover like urinary hydroxyproline, serum alkaline phosphatase reflect the actual status of bone resorption and bone formation over a short time-frame. Thus, they can be used as an adjunct to clinical and radiological evidence of fracture healing. A statistically

significant positive correlation between total urinary hydroxyproline excretion and serum alkaline phosphatase indicate progress towards satisfactory union. Indications of malunion could be helpful in instating early interventional procedures.

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