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Study of Bone Health using the Biochemical Markers in Type II Diabetes Mellitus

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Running title: Bone Health & Type II Diabetes

Abstract: ***Objective: To study bone health in a diabetic individual using the biochemical markers. Research Design and Methods: 125 subjects were included in the study. Dividing into two group i.e. group I included type II DM (n=75), group II was age matched control (n=50). The study was conducted at Dhiraj Hospital, Piparia, Vadodara, Gujarat, India. The protocol of the study was approved by ethical committee of SumandeepVidyapeeth.HbA_{1C}, Serum Calcium, Serum Phosphorous, Serummagnesium, Serum TRAP, Serum Osteocalcin levels were estimated. Results: There is a highly significant ($p<0.0001$) increase in the levels of HbA_{1C} in type II DM when compared with the normoglycemic subjects. The levels of the TRAP is significantly increased ($p<0.0001$) in typeII DM than its respective controls while Osteocalcin has been significantly low ($p<0.0001$) in type II DM when compared to controls. The minerals Calcium and magnesium was also significantly low in DM ($p<0.0001$) while no significant difference was observed in the case of phosphorus levels between both the groups. Conclusion: Diabetes mellitus is definitely affecting the bone health of an individual and that bone markers could a very promising tool to asses bone health.***

Keywords: Diabetes, biochemical markers, TRAP, Osteocalcin

1. Introduction

Bone is a specialized connective tissue which is in a dynamic metabolic state .It consists of three components: an organic matrix (called osteoid), bone mineral, and bone cells. The bone matrix is made up of type I collagen (90%) and the remaining 10% consisting of other proteins such as osteocalcin, osteonectin , and osteopontin.[1].This extensive extracellular matrix has a unique ability to become calcified, thereby forming, in conjugation with key component of the skeletal system i.e. the cartilage. It performs many important functions, including providing support to the body, protection of internal organs, acts as reservoir for minerals and providing sites of attachment for muscles, cavities for bone-forming cells.

There are two basic type of bone: cancellous bone and cortical bone. Bone is subjected to stress damage like any other tissue during the daily activities and eventually this leads to fracture as bone sustains microfracture and fatigue damage .This generally does not affect due to the characterized two opposing processes: formation and resorption called as “bone remodeling” where in old bone tissues are removed (resorption) and replaced by new bone tissues (formation) [2]. This process of remodeling is very essential for bone health. It begins with resorption of the old bone by osteoclasts, followed by the formation of the new bone by osteoblasts. It is observed that after the age of 30 - 40 years, there is decreased activity of osteoblasts, decreased production of growth factors and bone matrix which decreases through the rest of life, leading to osteoporosis. The cancellous bone is more metabolically active and more rapidly remodeled than cortical bone. The process of bone remodeling (resorption ► matrix synthesis► to mineralization) normally takes about 8 months as it is a slow but constant process. Typically, diseases of bone are characterized by an alteration in the bone resorption/formation balance. At the same time there are diseases like diabetes, osteoporosis, rheumatoid arthritis, renal osteodystrophy, cancer etc. which are said to affect the bone health. India has about 60 million adults having osteoporosis and approximately 2-3 million cases are being added annually [3] at the same time a huge number of diabetic population [4] hence it becomes important that an easy and cost effective techniques to monitor the bone status exists. Generally the bone status can be understood by a variety of technique, including histomorphometry, densitometry and measurement of calcium fluxes. Histomorphometry is invasive, expensive, has a long turnaround time, and is limited to a single skeletal site I iliac crest. Densitometry is precise and noninvasive but slow to reveal changes .It provides information on bone mineral content, bone macrostructure, integrity, quantity and fragility but it fails to inform about the rate of bone remodeling while measurement of calcium fluxes is technically difficult. While the biochemical markers of bone remodeling are noninvasive means of complimenting these techniques listed above and provides direct information about the current metabolism of the whole skeleton. So the changes in bone can be understood earlier by using these biochemical markers [5].These bone markers are the metabolites of bone remodeling present in urine and serum. They are classified into the following groups: a) enzyme activity markers of bone formation (reflect activity of osteoblast) and of bone resorption (reflect activity of osteoclast),b) bone matrix proteins and resorption products of organic skeletal matrix, which are released into circulation during bone formation and resorption; and c) inorganic skeletal matrix markers (calcium, phosphorus).(Table 1) They have helped increase our understanding of the pathogenesis of skeletal disorders, bone remodeling cycle, and the response of these disorders to therapy [6,7].They appear to be promising tools for defining the skeletal status over two decades. In fact bone turnover

markers along with other risk factors for osteoporotic fracture, may be used to define fracture risk and intervention thresholds [8,9].

If Diabetes has been associated with increased risk of fracture like stated by some researchers [10-13] then we need to think of the burden this disease is going to cause because fracture treatment is expensive, and rehabilitation not always successful, effective prophylaxis offers the only hope of alleviating the enormous social burden of hip fractures so we thought of this study to understand the bone remodeling cycle in the diabetic individual with the help of biochemical marker of bone turnover. This could be a non invasive and cheap technique for the early detection of bone fracture.

2. Research Design and Methods

2.1 Subject selection

The study enrolled 125 subjects. The control group comprising of 50 healthy subjects who were sex and age matched were included in the study. At the same type the test group comprised of 75 subjects who were diagnosed as type II DM. They were diagnosed as diabetic on the basis of the World Health Organization Criteria i.e. symptoms of diabetes plus random blood glucose concentration ≥11.1mmol/l(200mg/dl) or fasting plasma glucose ≥ 7mmol/l(126 mg/dl) at more than one occasion. The study was conducted in DhirajHopital, Sumandeep Vidyapeeth ,Piparia, Vadodara, Gujarat, India. No subject was included who had a previous history of stroke, myocardial infarction or any medical condition known to affect bone metabolism. The study protocol was approved by the Ethics Committee of the Institution (Sumandeep Vidyapeeth) and all the subjects included in the study volunteered after proper consent taken. All ethical norms were followed during the study.

2.2 Collection of specimen

Blood sample:
After 12 hrs of fast, venous blood sample was collected in different bulbs under aseptic conditions. EDTA bulb was used for glycated hemoglobin while Plain bulb was used for estimation of TRAP (tartarate resistant acid phosphatase) ,serum Osteocalcin, serum Calcium, serum Phosphorous, serum Magnesium . The blood was allowed to clot for 30 min at room temperature and then centrifuged at 2500g for 10 min at 4°C. Serum samples were then aliquoted and frozen at -70°C and then assayed in batches.

Biochemical markers:
Glycated hemoglobin (Accucare Ion Exchange Resin method), serum calcium (Accucare), serum phosphorous (Accucare), TRAP (Aspen Laboratories), Osteocalcin (Bio Line Osteocalcin ELISA Kit) and Serum magnesium was estimated by manual methods using Titan yellow[14].

Statistical analysis
All results are expressed as mean ±S.D. Students "t" test was used to compare the continuous variables between groups. Pearson's correlation was employed to calculate the 'r' value. Statistical significance was defined as $p<0.05$. The statistical analysis was done using SPSS version 14.0

3. Results

The study group was divided into two groups. The group I (n=75) was the type II diabetic individuals while the group II (n=50) consisted of control/healthy individuals. Table 2 summarizes the demographic data of participants in both the groups. There was no significant difference between the participants of both the groups with regards to age, sex, educational status, occupation, history of smoking and drug abuse etc.

Laboratory findings of all the patients are summarized in table3,4. Table 3 showsthat HbA1C is very significantly increased ($p<0.0001$) in group I as they are diabetic subjects. The Biochemical markers of bone resorption (Serum TRAP) levelswere significantly increased($p<0.0001$) in group I than group II while the level of bone formation marker i.e. Serum Osteocalcin has significantly decreased ($p<0.0001$) in diabetic subjects. Serum Calcium and Serum Magnesium levels were all significantly high ($p<0.0001$) in the control group than the diabetic individual while the level of serum Phosphorous was slight high in the control than the test group (group I) a significant difference was not observed.

From regression analysis, it was observed that there is a different type of correlation between HbA_{1C} and other parameters in the control and test group. As indicated in table 4 there is a negative correlation between HbA_{1C} and other parameters in the test group while a positive association was observed in the control. A very significant negative correlation was observed between HbA_{1C} and serum osteocalcin, serum TRAP, serum phosphorous, serum calcium.

4. Discussion

Diabetes and osteoporosis coexist is the finding of numerous studies [15,16]. This is because the process of bone remodeling is necessary to maintain bone strength and this is disarranged by diabetes. There are studies which have suggested that change in bone mass over time correlates with the concentration of markers [17]. Bone Markers can also be used in both localized disorders of bone turnover, such as Paget disease and cancer metastases, or in generalized disorders of bone modeling, such as osteoporosis or osteogenesis imperfect [18]. They can be used signally or a combination of markers can be used. The biochemical markers that were studied are Osteocalcin and TRAP (tartrate resistant acid phosphatase).

TRAP (Acid phosphatase) is a lysosomal enzyme found in bone, prostate, platelets, erythrocytes and spleen. Of the five isozymes of acid phosphatase, the bone isoform is tartrate resistant (TRAP)[19]. Initially organ cultures of newborn mouse calvaria were used to test the hypothesis that tartrate-resistant acid phosphatase might serve as a biochemical marker for osteoclast function. When bone resorption was stimulated in vitro with either parathyroid hormone or 1, $25(OH)_2D_3$, there was a significant increase in both tartrate-

resistant and tartrate-sensitive acid phosphatase activity in the medium relative to cultured controls. Tartrate-resistant activity was localized histochemically primarily over the osteoclast. The tartrate-sensitive activity was found primarily associated with bone cells other than the osteoclast. The results obtained from biochemical assays, histochemical observations, and polyacrylamide gel electrophoresis suggest that bone resorption in vitro results in the release of tartrate-resistant acid phosphatase from osteoclasts .Tartrate-resistant acid phosphatases of bone may be suitable biochemical probes for osteoclast function [20]. In the present study it was observed that level of this marker was significantly increased ($p<0.0001$) in diabetic individuals (2.9±2.6 IU/L) than the control (1.2±0.8).

The bone formation marker - Osteocalcin is a byproduct of bone matrix synthesis. It is the major noncollagen protein of bone matrix and is made up of 49 amino acid protein that is rich in glutamic acid (GLA) [21]. It is also known as GLA protein and BGP. It is found in bone and dentin and is 'highly conserved', meaning that its sequence is almost identical among vertebrates.It is a vitamin k and vitamin D dependant protein produced by osteoblasts and is the most abundant and most widely studied of the non-collagenous proteins in bone In humans, the osteocalcin is encoded by the *BGLAP*gene [22,23]. Osteocalcin is secreted solely by osteoblasts and thought to play a role in the body's metabolic regulation and is pro-osteoblastic, or bone-building, by nature [24]. It is also implicated in bone mineralization and calcium ion homeostasis. The osteocalcin level has been low in our study (4.1±2.3ng/ml) compared to the control group which was 8.4±4.5ng/ml.It was interesting to observe that Akin Oet al also had same observation as ours they had reported the osteocalcin levels to be (4.44±3.53 ng/ml) [25].Yasuhiro Hamada et al had reported that the bone histology studies in humans and animals have demonstrated that decreased bone formation is a critical mechanism of bone mass reduction in diabetes [26]. Osteocalcin also acts as a hormone in the body, causing beta cells in the pancreas to release more insulin, and at the same time directing fat cells to release the hormone adiponectin, which increases sensitivity to insulin [27].There are other studies which have also observed that the osteocalcin levels were low in the diabetic individuals [28-31] and this has been interpreted to represent a reduction in bone formation. This may be due to the inhibition of osteoblast function due to impaired insulin secretion and increase in insulin resistance leading to hyperglycemia [32]. Thus in this study also it was observed that the bone markers have a significant things to tell like that was observed by others [33].

When bone markers are compared with the studies done using bone mineral density ,there are studies which have reported that type 2 diabetic patients have a risk for hip, proximal humerus and foot fracture despite the presence of high BMD[34].There are also studies which have shown no significant difference in BMD values in diabetic individuals [35-37] and there are the other reported studies which state that the BMD is low [38].Hence we can conclude that the high BMD observed in type 2 diabetic patients may not parallel bone quality and strength. This may be because of the anabolic effect that insulin exerts on the bone tissue. Type 2 diabetes is preceded by a period of insulin resistance, hyperinsulinemia which may confer a protective effect on BMD [39,40]. The levels of bone minerals like calcium, Phosphorous, Magnesium was studied. It was observed that the levels of serum calcium, Magnesium was significantly decreased ($p<0.0001$) in diabetic individuals as compared to the control while the level of serum Phosphorous showed no significant difference ($p>0.05$) between the control and the study group. This observation is in accord with the findings of other studies[41,42] where in they had observed an decreased level of calcium and magnesium while Heaney et al found no significant difference in the level of serum phosphorus in diabetics.[43] This decreased level of the two mineral has been associated with the hypercalciuria and hypermagnesiuria. This can be well understood by the correlation analysis (Table3) where in both these minerals show a negative correlation with HbA_{1C} .At the same time it is known that magnesium is transported inside the cell with the help insulin and it is a well known fact that diabetes is due to defective insulin.[44] Hence magnesium metabolism is also affected and this hypomagnesiumia is also associated with the bone [45, 46].

5. Conclusion

The bone markers seem to be a good reflector of the bone health as it is a known factor that the bone health is affected in diabetic individuals which is the finding of the following studies [47,48].And the most important thing to be kept in mind is that they are noninvasive, inexpensive and can be repeated often. They can be used to understand the major changes which occur in a short time. Markers are derived from both cortical and tubercular bones and hence they reflect the metabolic activity of the entire skeleton hence a need does arise to explore the great potential of these markers in clinical application.

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Conflict of interest: No conflict of interest to disclose.

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Table 1: List of Bone turnover markers

I) Markers of bone formation	Abbreviation	Sample used for analysis
A) Osteocalcin	OC	S
B) Undercarboxylatedosetocalcin	ucOC	S
C) Procollagen –I extension peptide i) N –Propeptide ii) C-Propeptide	 PINP PICP	S
D) Bone specific alkaline phosphatase	Bone-ALP	S
II) Markers of bone resorption		
A)Hyroxyproline	Hyp	U
B) Hydroxylysine	Hyl	U
C)Pyridinoline	PYD	U
D)Deoxy pyridinoline	DPD	U
E) C terminal and N terminal Telopeptide	CTX	S/U
F)N terminal Telopeptide	NTX	S/U
G)C telopeptide generated by matrix metallo proteinases	CTX-MMP	U
H)Hydroxylysine glycosides	Gal-Hyl/Glc-Gal-Hyl	S
I)Bone Sialoprotein	BSP	S
J)Tartarate Resistant Acid Phoshatase (TRAP)	TRACP	S

Abbreviations: S=Serum,U=Urine

Table 2: Demographic data of the diabetic individuals (group I) and healthy controls (group II)

Parameter	DM (group I)	Healthy controls (group II)
Age(years)	58±10	51±12
Gender (M:F)	45:30	27:23
Smoking status : Never smoked Ex- smoker	38 25	28 12
Current smoker	12	10
Alcoholism: Never consumed	36	36
Ex- consumer	20	12
Currently alcoholic	19	2

Table 3: Levels of $HbA_{1C,,}$ Serum Osteocalcin, Serum TRAP, Serum Calcium, Serum Phosphorous, Serum Magnesium group I &group II

S. No.	Variables	Group I (n=75)	Group II (n=50)	P value
1	HbA_{1C} (%)	9.1±1.3	5.4±0.4	p<0.0001*
2	Serum Osteocalcin(ng/ml)	4.1±2.3	8.4±4.5	p<0.0001*
3	Serum TRAP (IU/L)	2. 9±2.6	1.2±0.8	p<0.0001*
4	Serum Calcium (mg%)	8.9±0.7	9.8±1.1	p<0.0001*
5	Serum Phosphorous (mg%)	2.9±0.7	3.1±0. 6	p>0.05#
6	Serum Magnesium (mg%)	1.7±0.4	2.2±0.3	p<0.0001*

All values are mean ±SD. While p<0.0001 was considered to be highly significant. P>0.05# was considered to be not significant.*

Table 4: Correlation Analysis of HbA_{1C} with other parameters in Group I and group II

Sr. No.	Parameters	Correlation Coefficient(r)	
		Group I	Group II
1	Serum Osteocalcin(ng/ml)	-0.78*	0.03
2	Serum TRAP (IU/L)	-0.68*	0.1
3	Serum Calcium (mg%)	-0.28**	0.04
4	Serum Phosphorous (mg%)	-0.34*	0.03
5	Serum Magnesium (mg%)	-0.098	0.14

*Where * indicates p<0.0001 is highly significant and ** indicates p<0.001is significant.*