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A comparative study of bone related biochemical markers in postmenopausal women with and without diabetes mellitus

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Abbreviations:
BMD: Bone Mineral Density, WHO: World Health Organization, SD: Standard Deviation, IGF: Insulin like Growth Factors, DEXA: Dual Energy X Ray Absorptiometry, ALP: Alkaline Phosphatase, RBS: Random Blood Sugar, GHb: Glycated Haemoglobin

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Abstract
The impact of type 2 diabetes mellitus on postmenopausal osteoporosis is still incompletely understood. Considerable debate exists as to whether postmenopausal diabetic women have reduced bone marrow density or elevated bone mass. The objective of this research was to study and compare various bone related biochemical markers in normal postmenopausal women and type 2 diabetic postmenopausal women with the effect of severity of diabetes on bone turnover. Calcium, phosphorus, alkaline phosphatase, 24 hour urinary hydroxyproline, random blood sugar (RBS) and glycated hemoglobin were estimated and compare between 30 postmenopausal diabetic women and 30 postmenopausal non diabetic women and correlated with glycated hemoglobin. The postmenopausal diabetic women had significantly elevated RBS and glycated hemoglobin. No significant difference was observed in 24 hour urinary hydroxyproline levels between the two study groups. Calcium, Phosphorus and alkaline phosphatase didn't show significant difference. However, calcium was on the lower side of reference range in both groups. Urinary hydroxyproline, a bone resorption marker was higher in the diabetic postmenopausal women but in reference range. Its value correlated positively with serum calcium levels. Hence, urinary hydroxyproline can be used as an inexpensive bone turnover marker in rural setting.

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1. Introduction

1.1 Osteoporosis and its mechanism

Old age has forever haunted mankind with its own set of complications and associated diseases, causing ill health. Around sixty million women in India itself are above the age of 55 years. Most of them would spend almost one third of their life in the postmenopausal stage (Padubidri et al., 2008).

Osteoporosis is a systemic skeletal disease characterized by decreased bone mineral density (BMD) resulting in bone fragility and risk of fractures (Christiansen, 1998). The WHO (World health organization) ranks Osteoporosis second in the list of non-communicable global health care problems. WHO criteria for diagnosing osteoporosis being "A BMD that lies 2.5 standard deviations or more below the average value for young

healthy women (T score of < 2.5 SD)" (WHO, 1994).

In postmenopausal group of women the osteoporosis mainly occurs due to the cessation of estrogen hormone production. This leads to a negative bone balance causing a gradual bone loss (Miura et al., 1995). Osteoporosis is highly prevalent in India. Studies conducted by Handa (2003) have extrapolated that in India the number of osteoporotic patients will increase to around 36 million by the year 2013. Almost thirty percent of post menopausal women are osteoporotic at the hip, lumbar spine or distal forearm. By the age of 80 years, 70% of women are osteoporotic at the hip, lumbar spine or distal forearm (Melton et al., 1992). Genetic and environmental factors are the determinants of peak bone mass and these with the lack of estrogens at menopause causes increased bone turnover and an imbalance between bone resorption and bone formation (Ralston et al., 2009).

1.2 Impact of diabetes on bone

Diabetes is a chronic multi-systemic disease which can be regarded as a pandemic affecting around 150 million people worldwide. Its prevalence in the rural parts of India is less (2-4%) compared to urban areas (4-11.6%) (Park, 2008). Diabetes through multiple pathways can affect the bone and hence can be referred to as an unconventional risk factor for fractures in older women (Schwartz, 2003).

Chronic hyperglycemia of diabetes results in non-enzymatic glycation of various proteins and the same holds true for bone proteins and collagen, especially type I collagen. The principle source of energy for osteoclasts is glucose (Hofbauer et al., 2007). These factors along with effect of hyperglycemia on the local microenvironment and cytokines could impair the bone quality.

Studies have shown a reduction in bone mass in type 1 diabetes which is associated with increased fracture risk (Schwartz, 2003; Oz et al., 2006). It has been proved beyond doubt that type 1 diabetes reduces bone mass, thereby causing increased fractures. However with regard to effect of hyperglycemia on bone marrow density in type 2 diabetes, there have been conflicting results. The impact of diabetes on bone is still incompletely understood or clear. Studies conducted by Kwon et al. have shown postmenopausal diabetic women to have reduced bone mineral density (BMD) (Kwon et al., 1996). On the contrary several cross sectional studies done by Oz et al., Van Daele et al., and Akin et al. respectively have found diabetics with elevated bone mass (Oz et al., 2006; Van Daele et al., 1995; Akin et al., 2003). In view of these conflicting results it is imperative for health care providers to detect the osteoporotic changes in diabetic individuals and monitor the disease progression (Acharya et al., 2010).

Bone turnover is regulated by local cytokines, cell matrix interactions and systemic hormones, and hyperglycemia may affect any of these micro-environments that regulate bone turnover (Okazaki et al., 1997). Osteoporosis is reported as a complication of type 1 diabetes. It has also been shown that older women with diabetes have increased risk of fractures (Schwartz et al., 2001). In type 1 diabetes, the deficiency of insulin and IGF-1, leads to impaired bone formation, abnormal mineralization, abnormal bone micro architecture, increased fragility of the bone and reduced peak bone mass (Thrailkill et al., 2005).

In addition to insulin, pancreatic β cells produce other osteotropic factors, such as islet amyloid polypeptide (IAPP, also called amylin) and preptin of the calcitonin-gene-related peptide family. Production of these peptides is abolished in patients with type 1 DM, reducing their effects on the RUNX2 gene, thereby reducing osteogenesis (Hamann et al., 2011).

1.3 Bone turnover markers

The bone turnover markers concentrations may rise in metabolic bone diseases and during physiological processes such as fracture healing and growth spurts (Thomas et al., 2012).

The onus is on the early detection of bone loss in type 2 diabetic postmenopausal women so that evidence based therapy can be instituted such as good glycemic control, maintaining an optimum calcium, vitamin D intake, and weight-bearing exercise, thereby preventing fractures altogether (Acharya et al., 2010).

Multiple biomarker measurements are better compared to single marker, also these markers are easier to measure and cheaper (Sachdeva et al., 2005).

BMD assessment methods like DEXA have become a standard method in clinical practice (Eastell et al., 2011). DEXA has its own disadvantages. The mineral density gets

miscalculated with changes in positioning of the subject, anatomic variations like floating ribs. Artefacts like coins, clips should be excluded before analysis (Garg et al., 2013).

2. Objective of Research

There is an ongoing controversy regarding the effect of type 2 Diabetes on bone loss. This study was done to compare various bone related biochemical markers in normal postmenopausal women and type 2 diabetic postmenopausal women in a rural setup. This study also aimed to study the effects of duration and severity of Diabetes on bone turnover markers.

3. Experimental

3.1 Source of data and study design
This was a hospital based study carried out at R.L. Jalappa Hospital and Research Centre, Kolar, from February 2012 to March 2013. The study group consisted of 60 postmenopausal women. Cases (Group I) - 30 postmenopausal women with clinically diagnosed type 2 diabetes mellitus, Controls (Group II) - 30 postmenopausal women without diabetes mellitus.

3.2 Inclusion criteria
Postmenopausal women with clinically proven type 2 diabetes mellitus as cases and postmenopausal women without diabetes as controls were selected. Non surgical, physiological menopausal women were included.

3.3 Exclusion criteria
Women more than 70 years of age, women on hormone replacement therapy, subjects with hyperthyroidism or hyperparathyroidism, on steroid therapy, heparin or drugs that alter calcium and phosphate levels, renal failure patients, chronic alcoholics or tobacco users, Cancer patients or those with other systemic diseases.

3.4 Collection of data
After obtaining written informed consent from the subjects, 5 ml of blood was drawn under aseptic conditions. Serum was separated. Twenty four hour urine sample was collected after a 24 hour gelatin free diet as Gelatin rich /protein rich food is known to alter the urine hydroxyproline levels. The twenty four hour urine sample was collected with 6 N Hydrochloric acids as preservative. Clinical data was obtained from each case with respect to name, age, clinical history, habit of alcohol consumption, history drug intake. Internal and external quality control was strictly followed.

3.5 Parameters measured
Twenty four hour urinary hydroxyproline (bone resorption marker) was measured using Modified Neuman and Logan method using colorimeter (Mitoma C et al., 1959). Serum calcium by the Arsenazo III dye method, serum phosphorus by phosphomolybdate reduction method. Serum Alkaline phosphatise (ALP) by p-nitrophenyl phosphate method were estimated. To assess the glycemic control, RBS and Glycated hemoglobin (GHb) was measured. Random blood glucose by Glucose oxidase/ Peroxidase method. Glycated hemoglobin by Cation exchange resin method using colorimeter.

3.6 Statistical analysis
Mean and standard deviation was calculated in all subjects for urinary hydroxyproline, serum calcium, phosphorus, alkaline phosphatase, random blood sugar and GHb. Data was compared using independent ‘t’ test.

Correlation of urinary hydroxyproline with serum calcium, phosphorus and alkaline phosphatase was done using Pearson’s correlation. To assess effect of glycemic control on bone, GHb was correlated with the bone turnover markers. SPSS package version 14 was used with a ‘p’ value of < 0.05 taken as statistically significant.

4. Results

In our study the postmenopausal women with diabetes (group I) and without Diabetes mellitus (Group II) had significant differences in the RBS values and glycated hemoglobin, with a p value <0.001. The mean RBS in Group I was 231.43±113.69 mg/dl, and in non diabetics it was 104.7±18.4 mg /dl, as shown in table (1). The GHb shows significant difference between the two groups with a p value of < 0.001 evident from table (1). The group I GHb suggests that they were under fair control of their diabetic status (according to the method).

Table (1) shows bone resorption marker, the 24 hour urinary hydroxyproline levels in the diabetics was higher, compared to non diabetic postmenopausal women. The mean levels of 24 hr urinary excretion of hydroxyproline in Group I women was 37.12±6.5 mg/day and in Group II was 33.55±8.2 mg/day i.e. within the reference

range for the age group. However there was no significant difference observed in 24 hour urinary hydroxyproline levels with p value of 0.068.

The mean serum calcium values were 8.34±0.78 mg/dl and 8.58±0.62 mg/dl in Group I and Group II respectively. However, the calcium values in both groups were towards the lower limit of the reference range. The decreased dietary calcium intake or deficiency condition such as post menopausal osteoporosis and their lower socioeconomic status could be the reason for such a finding. The urinary hydroxyproline shows positive correlation with the serum calcium levels in diabetic group, chart (1). Rest of the biochemical bone parameters did not show significant changes in the diabetics in table (1).

Chart 1: Pearson's correlation of urinary hydroxyproline with serum calcium in postmenopausal diabetic women showing significance with p < 0.05

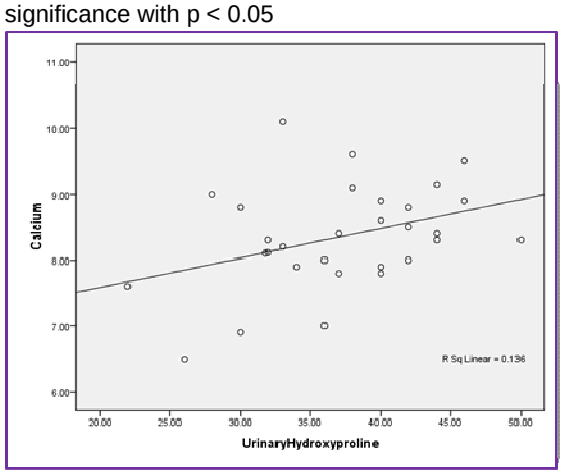

Table 1: Comparison of parameters between Diabetic and Non-Diabetic Postmenopausal Women

Parameter	Diabetic postmenopausal women n=30 (group 1) Mean+SD	Non-diabetic postmenopausal women n=30 (group 2) Mean+SD	'p' value	Reference range
Random blood sugar (mg/dl)	231.43+113.69	104.73+18.4	<0.001**	75-140
HbA1c% (glycated hemoglobin)	9.54+1.89	5.68+0.82	<0.001**	4-8
Urinary hydroxyproline (mg/day)	37.12+6.52	33.55+8.21	0.068	11.8- 42.5
Calcium (mg/dl)	8.34+0.78	8.58+0.62	0.206	8.8-10.2
Phosphorus (mg/dl)	3.75+0.81	3.64+0.75	0.584	2.5-4.8
Alkaline phosphatase (U/L)	120.16+33.71	116.26+35.93	0.666	100-290

5. Discussion

Our study is one of the first studies to be done on the effect of diabetes on postmenopausal bone loss in a rural setup. Significant difference was found between the two groups when RBS and glycated hemoglobin were considered with p values < 0.001. The Group I had a mean GHb value of 9.54+1.89 % whereas Group II had mean GHb of 5.68+0.82 %. These findings are consistent with the findings of some earlier studies (Sosa et al., 1996; Chen et al., 2013). Chen et al. (2013) found GHb, and fasting blood glucose to be higher in the diabetic group with p value <0.05. In our study the diabetic postmenopausal women had their diabetic status under fair control according to the GHb method reference range (9.0-10.0%). Group I subjects were clinically diagnosed to be type 2 Diabetics, and were on oral hypoglycemic drugs or insulin therapy.

In our study Group I had higher bone resorption marker levels than Group II, however this difference was not statistically significant (p=0.068 for urinary hydroxyproline). Similar results for urinary hydroxyproline were found in studies done by Sosa et al and Chen et al with p value > 0.05. Probably the diabetic state doesn't alter the resorption rate in bone (Sosa et al., 1996; Chen et al., 2013).

No significant difference was found between the two groups when other bone markers such as serum alkaline phosphatase, serum calcium and serum phosphorus were considered. This finding correlated with studies done by Sosa et al. (1996). They had assessed the bony changes by biochemical and radiological parameters. They measured formation and resorption markers. But they could not find any difference in bone mass between two groups when assessed by standard methods of BMD estimation like DEXA and CT scan. They measured the BMD at the lumbar spine and hip, and concluded that the bone mass was normal in non insulin dependent diabetes mellitus patients (Sosa et al., 1996).

With respect to the serum calcium, the mean values we observed were found to be lesser in

diabetics (8.34±0.78 mg/dl) than non diabetics (8.58±0.62 mg/dl) but with a non significant p value of 0.206. Studies done by Chen et al. (2013) reported similar findings. They found significant decrease in the serum calcium levels in type 2 diabetics. However their study was done in males of ≥ 60 years of age with type 2 diabetes (Chen et al., 2013). Studies with type 2 DM postmenopausal women and postmenopausal women without diabetes as controls, a significant decrease ($p < 0.001$) in the serum calcium and phosphorus level was observed. They suggested that poorly controlled type 2 DM patients have relative hypercalciuria probably caused by osmotic diuresis associated with glycosuria thereby contributing to lowered serum calcium levels, as found in our study (Varma et al., 2005).

Several studies have shown unaltered calcium levels in diabetics compared to non diabetics (Sosa et al., 1996; Hamad et al., 2012). Hamad et al. (2012) have suggested that the relative availability of dietary calcium sources (milk, dairy products), even for those with limited income could compensate for any deficiency associated with diabetes (Hamad et al., 2012).

Some studies found similar alkaline phosphatase levels between type 2 DM patients and controls (Cakatay et al., 1998; Hamad et al., 2012). These findings tally with our study. According to Hamad et al. (2012) the multiple sources of ALP in the body could be the cause of such a finding.

Studies by Cakatay et al. (1998) showed no statistically significant difference in the serum calcium and phosphorus levels was found as well. In spite of the decrease in the Osteoclacin levels in the diabetic group no difference was observed in deoxypyridine levels. It was postulated that this could be due to Diabetes affecting only the formation phase, while the resorption phase remaining unaltered (Cakatay et al., 1998).

Another study showed no significant difference in the prevalence of osteopenia and osteoporosis between Type 2 diabetic women and non-diabetic subjects (Maghbooli et al., 2007). According to them the prevalence of diffused osteoarthritis at the vertebral site in diabetic women is higher than non-diabetic women and this can lead to falsely elevated BMD at that site. This could possibly explain the elevated BMD found in Diabetic women when compared to non-diabetics found in some studies done previously (Oz et al., 2006; Van Daele et al., 1995; Akin et al., 2003).

Studies by Shwartz et al. (2001) have found increased fracture risk in old diabetic women. The increased fracture risk in diabetics could be due to the neuropathy and ocular complications of long term diabetes, causing frequent falls. Most diabetics involved in our study were under almost full or at least partial control of their diabetic status due to oral hypoglycemic drugs and insulin therapy. Thus the effect of chronic hyperglycemia could have got relatively canceled.

Research Highlights

The bone resorption marker hydroxyproline was found to be higher in the diabetic group when compared to non diabetics, though p value was not significant. Rest of the markers didn't show much difference. This study showed that as hydroxyproline excretion increased, serum calcium increased, mostly due to leakage from the bone matrix. Thus urinary hydroxyproline can be used as an inexpensive marker for assessing bone loss in a rural setup, where facilities like DEXA scan are unavailable.

Limitations of the Present Study

The control and case group should have the same dietary intake of calcium. Their dietary habits should have been assessed, as non vegetarians and milk consumers tend to have higher serum calcium than vegetarians. Many subjects with diabetes in the study were already on treatment, either oral hypoglycemic or insulin, due to which the effect of chronic hyperglycemia could have been cancelled. The study on diabetics who are not on any treatment would have been more conclusive.

Recommendations

Once osteoporosis sets in and causes fractures, the management of the patient becomes very difficult. The prevention of osteoporosis is the best modality to deal with osteoporosis. The thrust of research should be on finding easier and cheaper ways of assessing bone resorptive changes that can be used in very basic laboratory setups. Also effect of diabetes on bone needs to be evaluated further, as it is a pandemic disease.

Funding and Policy Aspects

Priority should be given for osteoporosis research in diabetics as there are reports of increasing number of old individuals suffering from fractures. There is a need for detecting bone loss at early stages, so as to prevent osteoporosis and its dreadful complications.

Justification of Research

This study was taken to assess the effects of type 2 diabetes on bone loss in diabetic women. This study was also for assessing the utility of cheaper bone biomarkers which could detect osteoporosis in early stages, and help in monitoring its complications. Urine is an underused diagnostic tool. There is paucity of studies on bone biomarkers of urine, especially in the rural scenario.

Conclusion

• Urinary hydroxyproline in diabetic postmenopausal women was higher when compared to non diabetic group but in reference range. Its value correlated positively with serum calcium levels, showing that as bone resorption occurred, the calcium from bone got released, thereby increasing serum calcium levels. Hydroxyproline being a collagen degradation product can be used as an inexpensive and reliable tool for assessing bone loss.

Serum calcium was found to be towards the lower side of reference range in both groups indicating a need for supplementation of calcium in women of this age group.

• The study was inconclusive to comment on effect of diabetes on bone, and further studies need to be conducted. DEXA scanning facility was not available in our setup, so it could not be done.

• A possible factor contributing to these results is that all patients involved in this study were under partial control so that the effect of chronic hyperglycemia is relatively cancelled.

Author's Contribution and Competing Interests

Dr. Esha M was involved in concept design, literature search, sample collection, experimental work and drafting the manuscript. Dr. Shashidhar K.N. designed the concept and the study protocol, literature search, drafting the manuscript and oversaw the experimental work. Dr. Raghavendra P and Dr. Gudi were involved in the selection of cases from the hospital and overseeing the work. Dr. Harish was involved in drafting the manuscript and statistical analysis.

Competing interests

None declared

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