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STUDY OF SOME COMMON BIOCHEMICAL BONE TURNOVER MARKERS IN POSTMENOPAUSAL WOMEN

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ABSTRACT

Markers of bone formation in serum include total and bone specific alkaline phosphatase, osteocalcin and Type 1 collagen carboxy terminal extension peptide. Bone resorption can be assessed by measuring plasma tartarate resistant acid phosphtase and urinary excretion of collagen degradation products: hydroxyproline, hydroxylysine glycosides and more recently the pyridinium crosslinks and associated peptides. We compared the excretion of hydroxyproline in women of reproductive age group to those of menopausal age group and found a significant difference in the two age groups. Urinary hydroxyproline was found to be significantly raised in post menopausal women. Thus hydroxyproline maybe used as the earliest indicator in the prognostic assessment of postmenopausal women of their risk of developing osteoporosis and fracture.

KEY WORDS

hydroxyproline, osteoporosis, postmenopausal women

INTRODUCTION

Bone is a dynamic tissue that is being remodelled constantly throughout life.It is composed primarily of the inorganic minerals (calcium and phosphate) and an organic matrix (type I collagen). There are two main types of bone cells i.e., osteoclasts and osteoblasts.Bone cells participate in the growth, modelling and remodelling of bone although they account for only a small fraction of bone volume.[1]

Organic matrix consists principally of collagen (90%), other matrix proteins and proteoglycans. It is rapidly mineralized by osteoblasts in close apposition to and throughout the collagen fibrils.[2] Despite its seemingly static appearance, bone is a remarkably labile tissue.Rate of formation or degradation of the bone matrix can be assessed by measuring the enzymatic activity related to the bone forming or resorbing cells. Bone matrix components are released into the circulation, either by the osteoblasts or by the osteoclasts .[3]

Bone formation is an orderly process in which inorganic mineral is deposited in relation to organic matrix. During bone resorption first calcium and phosphorus are released into the extracellular fluid and organic matrix is then resorbed. The concentrations of calcium, phosphate and magnesium in plasma are dependent on the net effect of bone mineral deposition and resorption, their intestinal absorption and renal excretion. Parathyroid hormone (PTH) and 1, 25-dihydroxycholecalciferol (calcitriol) are the principal hormones regulating these processes.[2] After 40-50 years of age, cortical bone is lost at a rate of about 0.3-0.5% per year in both the sexes. An accelerated loss of cortical bone is superimposed on age related loss around menopause.[4]

The menopause is the consequence of the exhaustion of ovarian follicles which results in decreased production of estradiol and other hormones. Osteoporosis is the term used for diseases that cause a reduction in the mass of bone per unit volume and is one of the dreaded afflictions of aging.[5] There is a close relationship between estrogen deprivation and its development. Several other factors like muscle bulk, body weight, malabsorption, smoking, alcohol and genetic factors also affect density of the bones.[4] Estrogen deprivation is suggested by early development of osteoporosis in women with premature menopause due to either natural or surgical cause.[5]

Much of the knowledge gained in the last decade on osteoporosis and other metabolic bone diseases has come from three different approaches: bone density, bone biopsy, and biochemical assays. Each approach has advantages and disadvantages. Density measurements are noninvasive, site specific, and are sufficiently sensitive to measure changes in bone density. However they are expensive, have a limited

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availability, and are unable to identify early changes resulting from therapy.[6] Bone biopsy is an invasive procedure, hence biochemical assessment of skeletal metabolism holds great importance. These markers reflect alterations in bone remodeling much earlier than they are apparent radiographically. In addition these markers now make it possible to determine the efficacy of anti-resorptive drugs, their optimum dosage in a time frame that is reasonably much less as compared to months before there is a radiographic evidence of a therapeutic response. They have untapped potential in the evaluation of patient at risk for accelerated bone loss e.g. in postmenopausal women.[7]

Most of the traditional and new markers of the bone resorption measure the collagen degradation products from osteoclast activity and these include urinary hydroxyproline, hydroxylysine and its glycosides, total or free pyridinoline crosslinks and crosslinked N or C telopeptides. Other markers of bone metabolism include enzyme levels in the serum that arise from osteoblast or osteoclast activity; namely bone specific alkaline phosphatase (ALP) (osteoblasts) and tartarate resistant acid phosphatase (osteoclasts).[2]

This study was undertaken to diagnose at the earliest the osteoporotic changes in the normal menopausal women by the easily available, reliable and cost effective colorimetric methods.

MATERIAL AND METHODS

The present study was conducted in the Department of Biochemistry with assistance from Department of Gynaecology and Obstetrics, Pt. B.D. Sharma PGIMS, Rohtak, in 25 healthy premenopausal women volunteers (25-40 years) and 25 healthy postmenopausal women in the age group of 55-65 years attending the outpatient Department of Gynaecology and Obstetrics.Subjects with history of smoking and alcohol intake, chronic illness in the past, surgically induced menopause and those on hormone replacement therapy (HRT) were excluded from the study

The inclusion criteria consisted of clinically normal subjects with same dietary status. The subjects of the two groups were compared using body mass index.

Five ml of venous blood was collected aseptically from antecubital vein and serum was separated. Serum proteins (Biuret Method)[8], Albumin(Bromocresol GreenMethod)[9], Calcium (OCPC Method),[10] Ionised Calcium (ISE),[1] Phosphorus (Fiske and Subbarow)[11]and Alkaline Phosphatase (King and Armstrong)[12] were estimated.

For estimation of urinary hydroxyproline(Modified Neuman and Logan),[13] calcium(OCPC)[10] and creatinine(Jaffe's Reaction)[14] 6 hours urine collection was done after adding a preservative (HCl: 5 ml/l of urine).The results were subjected to statistical analysis.

Mean and standard deviation for all the parameters (serum and urine) were calculated by applying unpaired 't' test.

Determination of Urinary Hydroxyproline: (Modified Neuman and Logan Method)[37]

Principle: Hydroxyproline is treated with $CuSO_4$ and H_2O_2 in an alkaline solution; this results in the formation of Δ pyrroline-4-carboxylic acid, which upon acidification is converted to pyrrole-2-carboxylic acid. The latter condenses with p-dimethylaminobenzaldehyde to give the coloured complex which is measured at 540 nm.

Reagents:

(i) Copper sulphate (0.01M): Dissolve 0.159 g of $CuSO_4$ in 100 ml D/W.

(ii) Sodium hydroxide (2.5 N): Dissolve 10 g of NaOH is 100 ml D/W.

(iii) 6% hydrogen peroxide.

(iv) Sulphuric acid (3N).

(v) p-dimethyl aminobenzaldehyde (5% solution in n-propanol).

(vi) Hydroxyproline standards: They are prepared which correspond to 5, 10, 15, 20, 25, 30, 35, 40, 45 µg of hydroxyproline.

Procedure:

Reagents	Test	Standard	Blank
1.Urine	1 ml	-	-
2.Standard	-	1 ml	-
3.Distilled water	-	-	1 ml
4.$CuSO_4$ (0.01M)	1 ml	1 ml	1 ml
5.NaOH (2.5N)	1 ml	1 ml	1 ml
6.6% H_2O_2	1 ml	1 ml	1 ml
7.H_2SO_4 (3N)	4 ml	4 ml	4 ml
8.p-dimethyl aminobenzaldehyde	2 ml	2 ml	2 ml

Mix and wait for 5 minute. Placed in a water bath at 80°C for 5 minute. Cool it in ice followed by keeping in water bath at 70°C for 16 minutes. Read the color at 540 nm.

Calculation:

The concentration of hydroxyproline in the samples was read directly from the standard curve.
Normal range:

18-25 years —> 14.0-38.7 mg/24 hr

20-77 years —> 11.8-42.5 mg/24 hr

RESULTS AND DISCUSSION

In our study duration of menopause was variable. Twenty eight percent of cases were in the early menopause i.e. 5-10 years. Majority of cases (48%) were postmenopausal since 10-15 years. In rest of the cases duration of menopause was more than 15 years increased bone remodeling was a characteristic feature of the immediate postmenopausal period.

In our study 28% of cases and controls were underweight i.e. had BMI <19.1; 64% of controls and 44% of cases had a normal BMI of 19.1-25.8 and 8% of controls and 28% of cases were overweight Majority of the controls (80%) and the cases (88%) were vegetarians. Diet has also been proven to be an independent risk factor for the development of osteoporosis. High protein diet (non-vegetarian diet particularly) leads to excessive acid formation which may contribute to "dissolution" of bones as the body tries to buffer the extra acid. Acidosis may also increase osteoclastic function directly.

Table 1 shows the comparison of various Biochemical parameters showing a significant increase in Alkaline Phosphatase in the cases.

Urinary hydroxyproline, the most performed measure of bone resorption, has the longest history of use.[2] In our study the excretion of hydroxyproline was increased in postmenopausal women as compared to the premenopausal women. This increase was highly significant statistically ($p < 0.001$) as shown in Table

TABLE 1. Comparisons of biochemical parameters

Total Ca^{2+} (mmol/l)	2.29±0.16	2.26±0.18	NS
Ionic Ca^{2+} (mmol/l)	0.97±0.11	1.02±0.08	NS
Total phosphorus (mmol/l)	1.32±0.16	1.40±0.29	NS
Alkaline phosphatase (μKat/l)	0.88±0.31	1.07±0.36	<0.05
Total protein (g/l)	64.48±3.40	66.48±4.89	NS
Albumin (g/l)	33.4±2.56	39.84±3.23	<0.001

TABLE 2. Comparisons of parameters in urine

Parameters	Controls (Mean±SD)	Cases (Mean±SD)	p value
Calcium (mg/24 hrs)	124.42±17.78	104.55±23.86	<0.001
Hydroxyproline (mg/24 hrs)	18.00±2.55	25.79±2.44	<0.001
Creatinine (mg/24 hrs)	1250±179.37	780.2±130.13	<0.001

TABLE 3. Excretion of hydroxyproline and calcium on the basis of BMI

	BMI (<19.1)			BMI (19.1-25.8)			BMI (>25.8)		
	Control (Mean±SD)	Cases (Mean±SD)	*p* value	Control (Mean±SD)	Cases (Mean±SD)	p value	Control (Mean±SD)	Cases (Mean±SD)	p value
Hydroxyproline /creatinine (mg/g)	15.22± 4.14	35.07± 7.66	<0.001	14.67± 3.29	32.32± 5.91	<0.001	14.53± 5.83	35.04± 3.66	<0.001
Calcium/ creatinine (mg/g)	105.98± 20.27	144.27± 36.10	<0.05	100.7± 22.87	137.06± 29.81	<0.001	93.5± 3.46	127.85± 32.83	NS

2.This increased excretion is due to increase in bone loss. And this was a characteristic feature of the immediate postmenopausal period.

Table 3 shows excretion of hydroxyproline and calcium on the basis of BMI Increased hydroxyproline / creatinine ratio in post menopausal women indicates increased bone loss. Increased calcium / creatinine ratio was observed in post menopausal women as compared to controls and this increase is statistically significant in subjects upto BMI <25.8($p < 0.001$). (BMI >25.8 , the change was insignificant)

Low BMI has been known to be a risk factor for osteoporosis. In our study, on the basis of BMI in the cases, not much difference is seen in the above ratios. There was increased loss of bone density during 5-6 years immediately following menopause due to sudden drop in estrogen levels and then reaching a plateau phase in which the bone loss attains almost a constant rate with a little variation as seen in our study where most of the cases are postmenopausal since 10-15 years.[15]

Thus measure of urinary hydroxyproline is an useful index of bone resorption in postmenopausal women and hence will play an important role in decreasing the incidence of fractures commonly observed in middle aged and elderly women by supplementing calcium or giving the patient HRT.

Any single measurement of a single biochemical marker of bone turnover has limited utility in the individual person. Thus three important biochemical indices of bone turnover i.e. serum alkaline phosphatase, urinary excretion of hydroxyproline and calcium were used and were found to be significantly increased in postmenopausal women reflecting the increased bone activity (osteoclastic and osteoblastic) as compared to premenopausal women. [16]

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