BIOCHEMICAL MARKERS OF BONE TURNOVER IN POSTMENOPAUSAL OSTEOPOROSIS AND RELATION TO FEMALE SEX HORMONE ESTRADIOL

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KEY WORDS: BIOCHEMICAL MARKERS OF BONE TURNOVER, POSTMENOPAUSAL OSTEOPOROSIS, ANABOLIC EFFECTS OF ESTRADIOL.

ABSTRACT

Objective: To evaluate bone turnover in postmenopausal osteoporosis and its relation to female sex hormone estradiol, using biochemical markers of both bone formation and resorption.

Methodology: Thirty females suffering from postmenopausal osteoporosis were studied compared to ten apparently healthy females. Estimation of biochemical markers of bone formation including procollagen type I carboxy-terminal propeptide (PICP), osteocalcin and alkaline phosphatase was performed. Hydroxyproline was estimated in the urine as a marker of bone resorption. Also, serum estradiol values were assessed.

Results: our results revealed an increase in the values of biochemical markers of bone formation PICP and osteocalcin, but no change in alkaline phosphatase was detected. Also, there was an increase in the biochemical marker of bone resorption, hydroxyproline. There were decreased estradiol values in our patients that were correlated with the biochemical markers of bone turnover.

Conclusion: Regular estimation of biochemical markers of bone turnover may be valuable in early diagnosis, prevention and treatment of postmenopausal osteoporosis. Estradiol strongly affects bone turnover in postmenopausal osteoporosis that reflected on the values of biochemical markers of bone turnover.
INTRODUCTION

Osteoporosis is a heterogeneous disease characterized by low bone mass and an increased risk of fractures. It is a major public health problem occurring primarily among the postmenopausal population. It is generally accepted that the rate of bone loss from all skeletal sites accelerates during the first postmenopausal years and subsequently declines. This accelerated postmenopausal bone loss is associated with an increase in bone turnover (Dequeker, 1997).

Bone turnover is characterized by the formation of new bone by the osteoblasts and the resorption of old tissue by the osteoclasts (Sergeni et al., 2001). The recent development of specific and sensitive biochemical markers, reflecting the overall rate of bone formation and bone resorption, has improved the non-invasive assessment of bone turnover abnormalities. Most of interest has been devoted to the use of these biochemical markers measurements in postmenopause, a condition in which modifications of bone metabolism cannot be detected by conventional markers of bone turnover like calcium and phosphorous assays (Garnero, 2001). Generally, bone osteoblastic activity was assessed by measurements of circulating osteocalcin, alkaline phosphatase and the C-terminal procollagen peptide (PICP) levels (Shiff et al., 2001) Bone resorption was assessed by measurements of urinary hydroxyproline. Reliable and convenient tests for quantifying bone turnover would aid in the clinical management of osteoporosis and other metabolic bone diseases (Delmas, 1992).

Procollagen type 1 carboxy-terminal propeptide (PICP) is a globular protein that is cleared from type I procollagen and then released into the circulation. Serum level of PICP reflects the synthesis of type I collagen. As PICP is produced by osteoblasts and is not incorporated into bone matrix, serum PICP levels have been considered as a marker of bone formation. This biochemical marker increases by 20% after the menopause. It correlates with indices of bone formation (Simon et al., 1984).

Osteocalcin is the most abundant non-collagenous protein in bone as it is produced only by osteoblasts and odontoblasts. About 75% of osteocalcin are incorporated in bone; the remainder circulates and is degraded by metalloenzyme in the liver and kidneys. Serum osteocalcin seems to reflect primarily a spillover of osteoblast synthetic activity rather than degradative products of resorption (Farrugia & Melick, 1986).

Hydroxyproline is an amino acid found mainly in collagens, where it accounts for about 13% of the total amino acid content, and is derived from proline by post-translational hydroxylation. Hydroxyproline is released
during bone resorption in the free form (90%) or peptide bound form. About 50% of hydroxyproline in urine is derived from bone (Deacon et al., 1987).

Estrogens are female sex hormones that affect calcium homeostasis and have beneficial effect on bone mass. They decrease bone resorption, and accelerate linear bone growth and epiphyseal closure in prepupertal girls. Long-term estrogen depletion is associated with loss of bone mineral content, an increase in stress fractures and postmenopausal osteoporosis (Kleeerekoper & Sullivan, 1995). Estrogen deficiency has clearly been identified as the major factor contributing to involutional osteoporosis in women, and the effects of estrogen on the skeleton have been the subject of intensive investigations (Doran et al., 1999). The main action of estrogen on bone remodeling is to decrease resorption. Estrogen deficiency causes bone loss by stimulating osteoclast formation (Manolagas, 2000).

**Aim of the work:**

This study was undertaken to evaluate bone turnover in postmenopausal osteoporosis by using biochemical markers for both bone formation and resorption to understand the role of estrogen in postmenopausal osteoporosis and bone turnover.

**PATIENTS AND METHODS**

This study was carried out on 30 postmenopausal females suffering from primary osteoporosis. Their ages ranged between 49 and 65 with a mean of 56.4 ± 0.66. They were diagnosed according to Gruker et al. (1984) Criteria. All patients had a natural history of menopause. We excluded patients who were taking medications that might affect calcium metabolism, patients who were suffering from hyperthyroidism, hyperparathyroidism, diabetes mellitus, renal or hepatic disease. Also patients who were under corticosteroids were excluded from the study.

A control group comprised 10 healthy females. Their ages ranged from 45 to 60 years with a mean of 51.8 ± 0.99.

**All subjects were subjected to:**

1. Full history taking
2. Thorough clinical examination with stress on the locomotory system.
3. Routine laboratory investigations including CBC, ESR, estimation of Calcium and Phosphorous using spectrophotometer (Nixon et al., 1986)
Assessment of biochemical markers of bone turnover included:

1) Assessment of bone formation (osteoblastic activity): by measurement of serum procollagen 1-C terminal propeptide (PICP), osteocalcin and alkaline phosphatase.

2) Assessment of bone resorption by measurement of urinary hydroxyproline.

3) Estimation of serum estradiol level.

Sample collection and preservation:

Fasting blood samples were collected from patients and controls in sterilized tubes and sera were obtained by centrifugation. Each serum sample was divided and kept at -20 °C. Few days before sample collections, patients and controls were instructed to eliminate food containing collagen from their diet like gelatin and gelatin containing foods as dietary collagen is catabolised and excreted in part as hydroxyproline containing peptide.

Twenty-four hour urine samples were collected, stored at -02 °C for estimation of hydroxyproline.

Determination of procollagen IC- terminal peptide:

It was measured with radio-immunoassay (Yoshida et al., 1997), the principle of which is that the sample containing an unknown amount of the substance to be assayed is mixed with a standard amount of a radioactively labeled derivative of the same substance. The labeled and unlabelled antigens are then allowed to compete for the limited number of high affinity binding sites of the antibody. The amount of radioactive antigen in the antigen antibody complex is inversely proportional to the amount of unlabelled antigen in the reaction mixture. After separating the free antigen from the antigen- antibody complex, the residual radioactivity is counted and the actual concentration is calculated with the aid of standard curve based on known amount of unlabelled antigen analyzed in parallel with the unknown.

Determination of serum osteocalcin:

It was measured by enzyme- linked immunosorbent assay (ELISA) (Delmas, 1988). This is an enzymatically amplified one step sandwich- type immunoassay. The standards and unknown diluted serum samples were incubated with anti-osteocalcin polyclonal detection antibody labeled with the enzyme peroxidase in microtitration wells coated with an affinity
purified anti-osteocalcin mouse monoclonal antibody. After incubation and washing, the wells were incubated with the substrate tetramethylbenzidine. An acidic stopping solution is added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measured at 450 and 620 nm. The absorbance measured is directly proportional to the serum concentration of osteocalcin. A standard curve of absorbance versus osteocalcin concentration is used from which osteocalcin concentration of the unknown samples can be calculated.

**Determination of urinary hydroxyproline:**

The urine samples were centrifuged and 200 ul of supernatant were added to 1 ml of saturated barium hydroxide solution. The samples were heated overnight at 100°C for complete hydrolysis. The hydrolyzed samples were cooled to room temperature; 50 ul of hydrochloric acid, 1 ml of borate buffer and 250 μl of chloramine T solution were added, left at room temperature for 25 minutes. 1 ml of Ehrlich’s reagent was added and incubation at 60°C for 15 minutes was done. Five hundred μl of sodium hydroxide and 3 ml of toluene were added and mixing for 10 minutes by rotatory mixer was done. Two ml of toluene were added after centrifugation for 5 minutes. Two ml hydrochloric acid were added and centrifuged for 5 minutes. Standard curve was plotted and the urinary hydroxyproline values were obtained from standard curves and multiplied by volume of urine to obtain the concentration of 24 hours urinary hydroxyproline (Podenphant et al., 1984).

**Determination of serum estradiol:**

Serum estradiol was measured by direct immunoenzymatic determination (Joshi et al., 1979). 17 β-estradiol (antigen) in the samples compete with horseradish peroxidase 17 β- Estradiol (enzyme-labeled-antigen) for binding into the limited number of anti- 17 β- Estradiol (antibody) sites on the microplates.

Statistical analysis was done according to Kirkwood (1989).
RESULTS

This study comprised 30 patients suffering from primary postmenopausal osteoporosis and 10 normal controls. The clinical data of both osteoporotic and control groups are summarized in table (1).

Table (1): Clinical data of patients & control groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Number</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>- Sex</td>
<td>females</td>
<td>females</td>
</tr>
<tr>
<td>- Age / years</td>
<td>49-65</td>
<td>45-60</td>
</tr>
<tr>
<td>Range</td>
<td>56.4 ± 0.66</td>
<td>51.8 ± 0.99</td>
</tr>
<tr>
<td>- Menopause duration</td>
<td>1-15</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>6.3 ± 1.32</td>
<td></td>
</tr>
</tbody>
</table>

Our results showed significant increase in serum calcium values in patients compared to controls \((p < 0.001)\). As regard to serum inorganic phosphorous, there was significant decrease in its values in patients compared to control \((p < 0.001)\). There was significant decrease in estradiol values in patients compared to controls \((p < 0.001)\) (Table 2).

Table (2): Ca, P and estradiol values.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>9.6-13.7</td>
<td>9.5-11.04</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean ± S.D</td>
<td>11.2 ± 0.3</td>
<td>10.4 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Phosphorous</td>
<td>3.4-5.9</td>
<td>4.2-6.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean ± S.D</td>
<td>4.07 ± 0.17</td>
<td>4.8 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Estradiol</td>
<td>54-74.2</td>
<td>112-350</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean ± S.D</td>
<td>63.8 ± 1.8</td>
<td>292.06 ± 14.9</td>
<td></td>
</tr>
</tbody>
</table>

The biochemical markers of bone turnover are shown in table (3). There was significant increase in PICP values in patients compared to controls \((p<0.001)\). Also there was significant increase in osteocalcin values in patients compared to controls \((p<0.001)\). However alkaline phosphatase values showed non significant change among patients and controls \((p>0.05)\). There was significant increase in urinary hydroxyproline values in patients compared to controls.
Table (3): Biochemical markers of bone turnover.

<table>
<thead>
<tr>
<th>Biochemical Marker</th>
<th>Patients</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Biochemical marker of bone formation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PICP</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Range</td>
<td>145-164</td>
<td>110-136</td>
<td></td>
</tr>
<tr>
<td>Mean + S.D.</td>
<td>151.9 ± 2.1</td>
<td>120.02 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>- Osteocalcin</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Range</td>
<td>5.9-12.8</td>
<td>4.9-10.9</td>
<td></td>
</tr>
<tr>
<td>Mean + S.D.</td>
<td>10.6 ± 0.8</td>
<td>8.15 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>- Alkaline phosphatase</td>
<td></td>
<td></td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Range</td>
<td>50-76</td>
<td>38-78</td>
<td></td>
</tr>
<tr>
<td>Mean + S.D.</td>
<td>61.1 ± 2.07</td>
<td>58.9 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>- Biochemical marker of bone resorption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Urinary hydroxyproline</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Range</td>
<td>39-75</td>
<td>31-67</td>
<td></td>
</tr>
<tr>
<td>Mean + S.D.</td>
<td>55.2 ± 2.6</td>
<td>41.1 ± 2.98</td>
<td></td>
</tr>
</tbody>
</table>

There was a negative correlation between serum estradiol and serum PICP, serum osteocalcin, serum calcium and urinary hydroxyproline. However there was a positive correlation between serum estradiol and serum phosphorous values (Table 4).

Table (4): Correlation between estradiol level and biochemical markers of bone turnover.

<table>
<thead>
<tr>
<th>Estradiol</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum PICP</td>
<td>0.798</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum osteocalcin</td>
<td>0.682</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum calcium</td>
<td>0.612</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urinary hydroxyproline</td>
<td>0.596</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum phosphorous</td>
<td>0.728</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Normally bone formation and bone resorption are coupled to keep a steady state bone turnover. Disturbances in bone turnover are detected by an increase or decrease in concentration of markers of bone formation and resorption. Serum and urine biochemical markers of bone turnover have been used to assess turnover states (**Rosen et al., 1997**) These markers seems to be sensitive for monitoring both severe pathology of bone turnover and slight physiological deficits in bone equilibrium beginning in
perimenopause (Morgante et al., 2001) Changes in bone turnover begin already in late premenopause in which bone formation may precedes increased bone resorption induced by estrogen withdrawal. These changes remain largely unchanged in early menopause (Klauss et al., 2002).

Our results showed increase in values of markers of bone formation including serum PICP and osteocalcin however no change in alkaline phosphatase was detected. These results were in agreement with Cheng et al., (1996) and Koga et al., (1999) who found higher levels of both formation and resorption markers of type I collagen. They suggested an increased rate of bone turnover and remodeling in osteoporotic elderly women. Shimozuma et al. (1999) reported that PICP influenced by age and menopause and found significant correlation between PICP and osteocalcin. Bouillon et al. (1992) found higher level of osteocalcin in postmenopausal women than in men and premenopausal women. Kushida et al. (1995) found that alkaline phosphatase, osteocalcin and PICP increase with age in healthy subjects and significantly increase in postmenopausal than in premenopausal. Also, Perry et al. (1998) found that osteocalcin was higher in postmenopausal than premenopausal subjects. Reeve et al. (1995) reported that serum osteocalcin rises slowly to a plateau at 5 years postmenopause. Vanderschueren et al. (1990) confirmed that serum measurements of osteocalcin reflect osteoblastic activity and thus bone formation, with stimulation of osteoblastic activity and bone turnover at the menopause.

Our results showed that the biochemical marker of bone resorption, urinary hydroxyproline values were significantly increased in postmenopausal osteoporotic females compared to healthy women. This was in agreement with Prince et al. (1995) who found that the hydroxyproline rises at menopause and remains elevated for the next 25 years. Our results were not in agreement with those of Wishart et al. (1995) who found that the levels of hydroxyproline fell with age. Scopacasa et al. (2000) reported that bone density was inversely related to urinary hydroxyproline. In contrast Reeve et al. (1995) reported that hydroxyproline fell progressively with time being consistent with diminishing rates of bone destruction which gradually re-equilibrated with bone formation as time passed after menopause.

Koga et al. (1999) found that serum alkaline phosphatase, osteocalcin, PICP and hydroxyproline were increased in women above 60 years suggesting that both formation and resorption were accelerated.

Our results showed a significant decrease in serum estradiol levels in postmenopausal osteoporotic women compared to healthy controls. There
was a negative correlation between serum estradiol values and serum PICP, serum osteocalcin, serum calcium and urinary hydroxyproline. This was in agreement with Khosla et al. (1998) who reported that serum estradiol levels decrease over the life span by 12% and bone resorption markers increase by 77%. Also, Fatayerji & Eastell (1999) found that estradiol decreases significantly with age by 33%. Goodman and Barret (1996) reported that serum estradiol decreases in women due to decrease in ovarian production and that estrogen therapy is associated with a decrease in markers of bone formation and resorption. Heshmati et al. (2002) reported that in the postmenopausal women the low serum estradiol levels exert a restraining effect on bone turnover and support that variation in these low levels may contribute to differences in the rate of bone loss. Naessen et al. (1997) reported that serum alkaline phosphatase and osteocalcin concentrations decrease in elderly women under potential estradiol suggesting reduced bone turnover. Robinson et al. (1996) were in agreement with our results. They found a negative correlation between serum estradiol and urinary hydroxyproline suggesting a role of estradiol in bone resorption. Estradiol decreases bone resorption by various mechanisms. Estradiol deficiency leads to release of calcium form bone, decreased serum PTH and decreased 1, 25 (OH) _2_ D_3_ and elevated serum calcium in elderly postmenopausal women (Chesnut et al., 1997). Falahati et al. (2000) reported that the role of estradiol and testosterone in maintaining bone formation may be through effect on inhibiting osteoblast apoptosis. Fatayerji & Eastell (1999) reported that sex hormones were positively correlated with bone turnover.

Conclusion:

-Biochemical markers of bone turnover provide a useful tool for evaluation of postmenopausal osteoporosis. So, regular estimation of these biochemical markers may help in early diagnosis, prevention and treatment of postmenopausal osteoporosis.

-Estradiol has a major effect on regulation of bone turnover that is reflected on the values of the biochemical markers of bone turnover.

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الدلاليات البيوكيميائية لإحلال العظام في مرض وهن العظام المصاحب
لسن اليأس وعلاقتها بهرمون الإستروجين

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إن مرض وهن العظام عند النساء في سن اليأس من المشاكل الصحية الهامة.
وقد كان الهدف من البحث تعين الدلالات البيوكيميائية المصاحبة لتجديد وإحلال العظام
في مجموعة من المريضات يعانين من وهن العظام بسبب انقطاع الطمث ومقارنتهم بمجموعة من
الأصحاء كمجموعة ضابطة.

وقد استعمل البحث على 30 مريضة يعانين من وهن العظام و10 وتم أخذ عينات دم
иبول وقد تم تعبيئة النهاية الكرويكسيلية للبروكولاجين 1، تعبيه مستوي الأوستيوكالسين وإنزيم
الفسفاتاز القولوي كدلاليات لتكوين العظام وتجددها في مصل الدم. وقد تم قياس نسبة الهيدروكسي
برولين في البول كدليل لإحلال العظام.

وقد تم تقييم مستوى الكالسيوم والفسفور وهرمون الإستروجين في مصل الدم.
وقد أسفرت النتائج عن زيادة ذات دلالة إحصائية في الدلالات الكيمية المصاحبة لتجديد
العظام في المرضى المصابين بهن العظام متناهية النهاية الكرويكسيلية للبروكولاجين 1
والأستيوكالسين ولكن لم يوجد تغير ذو دلالة إحصائية في إنزيم الفسفاتاز القولوي.
وجد زيادة في تركيز هيدروكسي برولین في البول (كمؤشر لإجلال العظام) في المرضى
المصابين بهن العظام.

وجد ارتفاع في نسبة الكالسيوم والفسفور في مصل الدم.

لوحظ وجود نقص في مستوى هرمون الاستروجين في المريضات يعانين من وهن العظام يناسب
مع الدلالات البيوكيميائية لتجديد وإحلال العظام.

وكانت خلاصة هذا البحث أن الدلالات البيوكيميائية تقوم بدور هام في تعديل وهن العظام
في سن اليأس. وأن قياسها بالتمائم سوف يساعد على التشخيص المبكر لهذا المرض ويساعد على
وضع وعلاجه مبكراً. وأن هرمون الاستروجين يقوم بدور كبير في تنظيم إحلال العظام ويعثر على
الدلاليات البيوكيميائية بشكل كبير.