The Effect of Folic Acid Supplementation on Osteoporotic Markers in Ovariectomized Rats.

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ABSTRACT

Menopause is considered to be a natural change of life, which may be accompanied by various health problems such as osteoporosis and fracture. This study is designed to assess the effect of estrogen treatment, folic acid supplementation on bone markers in ovariectomized (ovx) rats.

Fifty adult female rats were divided into five equal groups: one served as control, the rats in the second group were subjected to ovariectomy, the third group were exposed to ovariectomy and treated with estrogen, the fourth group were ovariectomized and supplemented with folic acid and the fifth group were consist of ovariectomized rats treated with estrogen and supplemented with folic acid.

Ovariectomized rats showed insignificant increase in serum ca+2 and osteocalcin levels with significant decrease in serum vitamin D3 (vit.D3) and significant increase in alkaline phosphates activity and homocysteine levels.

Estrogen treatment of ovariectomized rats showed a significant decrease in serum homocysteine (Hcy) levels, alkaline phosphates activity accompanied with insignificant decrease in serum ca+2, vit.D3 and osteocalcin than ovariectomized rats.

Supplementation of folic acid to ovariectomized rats induced significant increase in osteocalcin and decreased Hcy levels, insignificant changes in serum ca+2, vit. D3 and alkaline phosphates than ovariectomized control rats, while combined treatment of ovariectomized rats with estrogen and folic acid exhibit better action on bone turnover with marked decrease in Hcy level.

So, we can conclude that, supplementation of folate with estrogen treatment has a beneficial effect for menopausal women to avoid osteoporosis and fracture.

Keywords: osteoporosis, ovariectomy, fracture, homocysteine, bone markers, bone turnover, osteocalcin.

INTRODUCTION

Primary osteoporosis is an age related disorder characterized by decreased bone mass and increased susceptibility to fractures in the absence of other recognizable causes of bone loss, Zengin A. et al. (2010).

The risk of developing osteoporosis increases with age and is higher in women than in men. Among the many possible contributors to primary osteoporosis, estrogen and calcium deficiencies and they are considered to be the most important causes, Coxam V. (2005). During menopause bone remodeling is increased and agents that suppress bone resorption can stabilize bone mass, Canalis E. (2010). Estrogen is usually thought to maintain bone mass by inhibiting bone resorption and formation.

Estrogen deficiency at menopause increased bone turnover with loss throughout the skeleton. A number of theories have been proposed including imbalanced turnover at the level of basic multicellular unit of bone remodeling with a greater level of bone resorption relative to bone formation, Deal C. (2009).

In addition, the increase in plasma homocysteine levels with menopause suggests a close relationship between homocysteine metabolism and estrogen status, Bednarek-Tupikowska G. et al. (2005).
Recently, hyperhomocysteinemia (HHcy) has been suggested to have adverse effects on bone and HHcy in rats induces accumulation of Hcy in bone tissue that is accompanied by bone loss, Herrmann M. et al. (2009).

With mild to moderate hyperhomocysteinemia, increased bone resorption by osteoclasts may contribute osteoporosis with an impaired biomechanical quality of the healing bone, Claes L. et al. (2009).

Estrogen replacement therapy is claimed to reduce bone turnover, increase bone mineral density and decreases vertebral and hip fractures rates, lwamoto, J. et al. (2003) and Lindsay, R. et al. (2005).

Part of the risk for osteoporosis in women is established by their unhealthy life style in the premenopausal period, Vos, H. M. et al. (2010).

Folic acid supplementation reducing an elevated homocystein levels. So, folic acid and B vitamin may help to reverse the problems associated with hyperhomocysteinemia, Righetti, M. (2009).

So, the aim of this study was to evaluate the effect of ovariectomy, estrogen, folic acid supplementation on bone metabolism in intact and ovariectomized adult female rats by measuring indices of bone resorption and bone formation (bone biomarkers) and histopathological examination.

**MATERIALS AND METHODS**

The present study conducted on experimental animals.

In this work 50 mature female albino rats weighting 200±50 gm were housed under the prevailing atmospheric conditions allover the experimental period in the laboratory of physiology.

**Experimental procedure:**

Ovariectomies of rats were done for 40 rats as described by, Oh H.Y. et al. (2007) and Vos H.M. et al. (2010) rats served without ovariectomy (intact).

Rats were divided into equal groups after two weeks of ovariectomy as follow:

- **Group I:** Intact rats served as a control (I)
- **Group II:** Ovariectomized rats served as a control (II)
- **Group III:** Ovariectomized rats received daily 2mg of conjugated estrogen orally, Cagnacci, A. et al. (2006).

Group IV: Ovariectomized rats received normal diet fortified with folic acid powder in a dose 7.5 mg/day, Villa, P. et al. (2005).

Group V: Ovariectomized rats received daily the same dose of estrogen orally and maintained on the diet fortified with folic acid powder.

Blood samples will obtained from the retro-orbital sinus all over night fasted rats under light ether anesthesia with capillary tubes.

Blood immediately centrifuged and serum will collected and stored at -20’c. until assayed for estimation of the following:

1- Calcium ions concentrations.
2- Vitamin D3 levels.
3- Osteocalcin hormone concentrations.
4- Activity of alkaline phosphatase.
5- Homocysteine levels.

Also, Histopathological examination of bones will be done.

**RESULTS**

The present work revealed insignificant increase in serum C+2 concentration, significant decrease in the serum vit. D3 and significant increase alkaline phosphates activity with insignificant changes in osteocalcin level (Table 1 and Fig. 1). Also, we observed a relationship between elevated homocysteine level and bone markers of osteoporosis in ovariectomized rats (Table 2 and Fig. 2).

As regard to estrogen treatment, our study showed a significant decrease in serum Hcy, alkaline phosphates accompanied with insignificant decrease in serum ca+2, vit. D3 and osteocalcin level when compared to ovariectomized rats.

In this research supplementation of folic acid alone to ovariectomized rats induced insignificant increase in serum calcium level when compared to both control groups, insignificant decrease in vit. D3 and alkaline phosphates with significant increase in osteocalcin hormone in comparison to ovariectomized rats.

The present study proved that combined administration of conjugated estrogen with supplementation of folic acid exhibit better action on bone turnover and prevents the loss of bone caused by estrogen deficiency in ovariectomized rats.
The Effect of Folic Acid Supplementation on Osteoporotic Markers in Ovariectomized Rats

(1): Effect of folic acid administration on serum calcium (mmol/l), vit.D3 (ng/ml), osteocalcin (ng/ml) and alkaline phosphatase activity (u/l) in normal rats, Ovariectomized and Ovariectomized treated with estrogen.

Table 2: Effect of folic acid administration on serum homocysteine levels (Umol/L) in normal rats, ovariectomized and ovariectomized treated with estrogen.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Calcium</th>
<th>Vitamin D3</th>
<th>Osteocalcin</th>
<th>Alkaline Phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±S.E.</td>
<td>Mean±S.E.</td>
<td>Mean±S.E.</td>
<td>Mean±S.E.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Ta) significant test</td>
<td>(Tb) significant test</td>
<td>(Ta) significant test</td>
<td>(Tb) significant test</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2.681±0.057</td>
<td>-</td>
<td>105.60±2.420</td>
<td>0.000*</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td></td>
<td>3.186±0.470</td>
<td>0.110</td>
<td>61.926±6.614</td>
<td>-</td>
</tr>
<tr>
<td>Ovariectomized treated with estrogen</td>
<td></td>
<td>2.723±0.039</td>
<td>0.884</td>
<td>54.401±4.555</td>
<td>0.000*</td>
</tr>
<tr>
<td>Ovariectomized treated with folic acid</td>
<td></td>
<td>2.799±0.083</td>
<td>0.778</td>
<td>59.167±8.131</td>
<td>0.000*</td>
</tr>
<tr>
<td>Ovariectomized treated with estrogen and folic acid</td>
<td></td>
<td>2.371±0.028</td>
<td>0.737</td>
<td>81.363±6.950</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

The mean difference is significant at the 0.05 level.
(Ta): significant as compared with normal control group.
(Tb): significant as compared with Ovariectomized group.

Table 2: Effect of folic acid administration on serum homocysteine levels (Umol/L) in normal rats, ovariectomized and ovariectomized treated with estrogen.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Homocysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±S.E.</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>15.463±0.751</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td></td>
<td>23.075±1.114</td>
</tr>
<tr>
<td>Ovariectomized treated with estrogen</td>
<td></td>
<td>19.738±0.380</td>
</tr>
<tr>
<td>Ovariectomized treated with folic acid</td>
<td></td>
<td>11.338±1.721</td>
</tr>
<tr>
<td>Ovariectomized treated with estrogen and folic acid</td>
<td></td>
<td>9.243±1.238</td>
</tr>
</tbody>
</table>

The mean difference is significant at the 0.05 level.
(Ta): significant as compared with normal control group.
(Tb): significant as compared with ovariectomized group.
Histological Results:

In normal control group, histological examination of the cancellus and compact bone revealed normal architecture of bone cells and matrix (Figs. 3a & 3b).

On the other hand, ovariectomized rats showing an increased number of osteoclast cells with abnormal matrix in the cancellus and compact bone with cavity formation (Figs. 4a & 4b). Ovariectomized rats treated with estrogen alone or combined with folic acid revealed less degenerative changes with less number of osteoclast cells (Figs. 5a & 5b).

**DISCUSSION**

The data from this study showed that indices of both bone resorption and formation increased markedly after ovariectomy.

The insignificant increase in serum Ca+2 after ovariectomy may be due to enhancement of osteoclasts and increased number of mature osteocasts, with continuous release of calcium and phosphate as well as peptides from the bone matrix.

Our results are consistent with, Ahlborg, H.G. *et al.* (2001) who found specific differences in calcium homeostasis in osteoporotic subjects.

A negative calcium balance may be attributed to a primary process within the bone, with secondary consequences on the renal and gut handling of calcium or due to abnormalities in other organs and hormones regulating extracellular calcium homeostasis, mainly parathormon hormone PTH and osteocalcin hormone.

Also, (PTH) causes an increase of intestinal calcium absorption through its action on vit.D3. The active Ca+2 absorption in the duodenum is under the control of 1, 25 (OH2) D, and the vit. D3 metabolite increases the intestinal cell synthesis of Ca+2 binding protein which enhances the net absorption of Ca+2. PTH also decreases osteoblastic collagen synthesis, but osteoclastic bone resorption increases with a net increase of mineral release from bone into the extracellular fluid, Kim D.J. *et al.* (2006).

The study of, Lee Y.M. *et al.* (2005) reported that decreased estrogen level in female increased the sensitivity of bones to the action of PTH leading to bone resorption with lower bone mineral density, Krivosíková Z. *et al.* (2010).

In contrast, serum Ca++ level decreased in post menopausal women in the study of, Aspray T.J. *et al.* (2005).

Also, Xie F. *et al.* (2005) have reported a decreased serum Ca+2 after ovariectomy in rats and the increased bone resorption does not appear to occur but the process of bone turnover increased. The present work revealed significant increase in the serum Vit. D3 and alkaline phosphatase with insignificant changes in osteocalcin level in ovariectomized rats. This result may be explained by the increased bone resorption due to an increase sensitivity of bone to PTH as a result of decreased plasma estrogen after ovariectomy and increased level of Hcy, Herrmann M. *et al.* (2007).

Concerning the increased alkaline phosphatase and vit. D3 after ovariectomy indicates an increased osteoblastic activity on bone formation. In accordance, Arshad, M. *et al.* (2004), found a relationship between serum alkaline phosphatase and age, with marked rise after the first decade of menopause, also, Aspray T. J. *et al.* (2005), reported an increase in alkaline phosphatase activity after few days of ovariectomy.

The insignificant decrease in serum concentration of osteocalcin may be due to bone resorption after ovariectomy, Cardenas Morales B.E. and E. Perez Campos (2003) and Mizoguchi T. *et al.* (2007), found the same results after 9 weeks of ovariectomy. Also, Ozdem S. *et al.* (2007), found that bone formation marker (osteocalcin) decreased in rats with hyperhomocysteinemia (HHcy) due to bone resorption.

Inconsistent with our results, Hankenson K.D. *et al.* (2005), who found an elevation in osteocalcin concentration after 2 weeks of ovariectomy but its level decreased after five weeks and they explained these results by increased the process of bone formation.

Also, Seifert-Klauss V. *et al.* (2005), were observed increased serum osteocalcin in premenopausal women and
attributed that to increased bone loss before postmenopause as a result of increased ovulatory cycles.

The adverse effects of aging on the skeleton such as oxidative stress, are the fundamental mechanisms of the decline of bone mass and strength and osteocyte death is major contributor to the decline bone strength with age, Manolagas S.C. and A.M. Parfitt (2010). Studies in animals and humans suggest that homocysteine may weaken the collagen crosslink's and, if present in large amounts, interfere with bone remodeling, Herrmann M. et al. (2007) and Levasseur R. (2009).

In this study, we observed a relationship between hyperhomocysteinemia and the bone marker of osteoporosis in ovariectomized rats may be attributed to loss of estrogens that accelerates effect of aging on bone by decreasing defense against oxidative stress, Almeida M. et al. (2007).

Homocysteine directly activates osteoclasts formation and activity through increased generation of intracellular reactive oxygen species, Cagnacci A. et al. (2008). Also, elevated Hcy is associated with reduced bone mineral density especially with bone fracture. Golbahar J. et al. (2005), suggested that HHcy and low folate associated with osteoporosis in postmenopausal women. Findings of, Kim D. J. et al. (2006), revealed that high plasma Hcy induced apoptotic effect on osteoblasts.

The results of, Herrmann M. et al. (2005) demonstrated week, but significant relations between Hcy and markers of organic and inorganic bone resorption, suggesting a mechanistic role of Hcy in bone metabolism.

The data of the present study showed a significant decrease in serum Hcy, alkaline phosphatase accompanied with insignificant decrease in serum ca+2, vit. D3 and increased osteocalcin level after estrogen treatment to ovariectomized rats. This effect was explained by, Bednarek-Tupikowska G. et al. (2005), that estrogen have a profound influence on Hcy and lipid peroxides and concluded that Hcy level controlled by administration of hormone replacement therapy.

From other hand, Baines M. et al. (2007), reported that low serum folate is a significant risk factor for osteoporosis, and supplementation of folic acid may have an effect on the skeleton in postmenopausal women. While, Kim D.J. et al. (2006), suggest that methionine synthase reductase (MTRR) is an enzyme involved in the conversion of Hcy to methionine and they hypothesized that certain genetic polymorphisms of MTRR leading to reduced enzyme activity may cause hyperhomocysteinemia and affect bone metabolism in postmenopausal women.

Also, Cagnacci A. et al. (2003), found a major association between folate and bone mineralization and high dietary intake of folate exerts positive effect on bone mineral density. Recent studies in rodents indicate that aging and the associated increase in reactive oxygen species (Ros) are the proximal culprits because it influence the generation and survival of osteoclasts, osteoblasts and osteocyte and loss of estrogen decreases defense against oxidative stress in bone and this accounts for increase bone resorption associated with loss of estrogen so, estrogen treatment may improve bone formation. In addition, increased glucocorticoid production and sensitivity with advancing age decrease skeletal hydration and thereby increase skeletal fragility by attenuating the volume of bone vasculature and interstitial fluid, Manolagas S.C. (2010).

In the present research supplementation of folate alone to ovariectomized rats induced return the calcium level, vit. D3 concentration to the normal value, accompanied with significant increase in osteocalcin level and significant decrease in alkaline phosphatase activity. This data indicates an improvement in bone formation. This effects was explained by, Wolters M. et al. (2004), that folic acid act as coenzymes and show a close molecular interaction of the bases of the Hcy metabolism.

The data of, Rejnmark L. et al. (2008), are in agreement with our results, they reported a positive association between nutrient dense (intake of fruits, vegetable and whole grain) and bone health.In addition, Levasseur R. (2009),
suggest that folate supplementation to patients with HHcy may improve not only bone health, but also general health.

Also, Chen K.J. et al. (2005), suggest that poor folate and B6 has a synergistic effect on the risk of HHcy in elderly male and female. Also, the study of, Herrmann M. et al. (2009), on rats found that folate deficiency induced HHcy but has no effect on bone health in healthy adult rats.

The present study proved that combined estrogen and folic acid supplementation exhibit better action on bone turnover and prevent the loss of bone caused by estrogen deficiency in ovariectomized rats.

So, there is a possibility that folic acid could be added to estrogen as a HRT to be a novel anti osteoporosis drug for menopausal women.

Therefore, maintaining adequate folic acid should be emphasized as an important measure for reducing Hcy level among elderly people. Also, we recommended that administration of estrogen and folic acid has a beneficial effect to pre- and postmenopausal women to improve and prevent the occurrence of osteoporosis and fracture.

Acknowledgement

I wish to express my sincere gratitude and utmost thanks to the Dean of Scientific Research for funding this research.

REFERENCES


Figs. 3a & 3b: Photomicrograph in normal control rats, in the cancellus and compact bone showing normal architecture of bone cells and matrix (H. & E.x.125).

Figs. 4a & 4b: Photomicrograph in ovariectomized rats showing an increased number of osteoclast cells with abnormal matrix in the cancellus and compact bone with cavity formation (H. & E.x.125).

Figs. 5a & 5b: Photomicrograph in ovariectomized rats treated with estrogen alone or combined with folic acid showing less degenerative changes with less number of osteoclast cells (H. & E.x.125).
تأثير التغذية التكميلية بحمض الفوليك على دلالات هشاشة العظام في الجرذان مستأصلة المبايض

نورة صالح الصوبيان - نادية حلفي محمود
قسم البيولوجيا، كلية العلوم، جامعة القصيم، المملكة العربية السعودية.

يعتبر سن اليأس من التغيرات الطبيعية التي تحدث في الحياة، والتي قد تكون مصحوبة بمشاكل صحية عديدة مثل هشاشة العظام والكسور. وقد تم تصميم هذه الدراسة لتقديم تأثير العلاج بالاستروجين وكملاط حمض الفوليك على مؤشرات هشاشة العظام في الجرذان مستأصلة المبايض.

وقد تم تقسيم الجرذان الحمسون بالغة إلى خمس مجموعات متساوية: الأولى مجموعة ضابطة، والجموعة الثانية تعرضت لاستئصال المبايض وتمت مجموعات ضابطة ثانية، ت تعرض المجموعة الثالثة مستأصلة المبايض وتمت معاملتها ببهرمون الاستروجين، أما المجموعة الرابعة فقد تعرضت لاستئصال المبايض مع إعطائها حمض الفوليك، و المجموعة الخامسة مستأصلة المبايض وعوامل ببهرمون الاستروجين وضمنها حمض الفوليك. أظهرت مجموعة الجرذان مستأصلة المبايض زيادة غير معنوية في مستويات Vit.D3 في المصل، وارتفاع ذو دلالات معنوية في نشاط إنزيم الفوسفاتاز الفاعلي في مستويات ومستويات الھمون بين الجرذان مستأصلة المبايض بالاستروجين المتأثرين بال[Int.84] ويستنجد من النتائج على أن معاملة الجرذان مستأصلة المبايض بالإستروجينات المتأثرين على انخفاض معنوي في مستويات الھمون بين الجرذان مستأصلة المبايض ومستويات الفوليك والكسور، وارتفاع ذو دلالات معنوية في مستويات Vit.D3.

أما إضافة بودرة حمض الفوليك إلى الطعام الجرذان مستأصلة المبايض أدت إلى حدوث ارتفاع معنوي في مستويات Ca2+ في الجرذان مستأصلة المبايض وانخفاض معنوي في مستويات Ca2+ في المصل المستأصلة المبايض، ونماذج نتائج في مستويات Vit.D3 بين الجرذان مستأصلة المبايض وإضافة حمض الفوليك إلى الطعام جرذان مستأصلة المبايض أظهرت تأثير أفضل على عملية تحلل العظام مع انخفاض معنوي في مستويات الھمون.

هذا، يمكننا أن نستنتج أن العلاج بالبهرمون الاستروجيني مع إضافة حمض الفوليك للطعام له تأثير مفيد للسيدات المقبلات على سن اليأس لتجنب حدوث هشاشة العظام والكسور.