Oxidative Stress as a Risk Factor of Osteoporotic Model Induced by Vitamin A in Rats

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Abstract: The present study was undertaken to investigate the oxidative stress of vitamin A as a risk factor of osteoporosis. The osteoporosis was induced by intragastric administration of vitamin A at a daily dose of 80 mg/Kg b.wt. for 7 consecutive days (group 1), 14 consecutive days (group 2) and 21 consecutive days (group 3). Vitamin A administration revealed significant increase in serum ALAT, ASAT and ALP activities. Malondialdehyde (MDA) levels was significantly increased in both liver and the femoral bone at the three experimental periods which indicating an oxidative stress. In addition, the data recorded revealed marked decreases in the levels of catalase (CAT) and glutathione reduced (GSH) in the liver and bone tissues in comparison with the corresponding controls. Vitamin A, however, produced time dependent osteoporosis with an appearance of mild effect at the early days of the experiment. Moreover, results of structural bone histomorphometric analysis revealed that vitamin A exerted negative effects on structural trabecular bone parameters by reducing trabecular area (Tb. Ar) and trabecular thickness (Tb. Th), which, is consistent with the significant decrease in bone mineral density (BMD) of the femoral bone. Also, the levels of both Calcium and phosphorus were decreased in both serum and bone. In conclusion, hypervitaminosis A induced an oxidative stress in both liver and bone after the three experimental periods. An osteoporotic rat model was induced after only 7 days rather than 15 days.

Key words: Vitamin A- Osteoporosis-Oxidative stress-Femur- Liver

INTRODUCTION

Vitamin A is necessary for growth, steroid production and the immune system (Moro et al., 2008). The administration of vitamin A in high doses stimulates bone resorption and inhibits bone formation (Muthusami et al., 2005 and Lind et al., 2006). Short term administration of high dose of vitamin A causes diverse and variable clinical signs and produced a marked alteration of the activity of liver enzymes (Alarcon- Corredor and Alfonso, 2007). In addition, there is growing evidence that vitamin A intakes twice the current recommended dietary allowances is associated with hip fracture and osteoporosis (Penniston and Tanumihardjo, 2006 and Rothenberg et al., 2007).

Osteoporosis is a major metabolic bone disease, which results from a disturbance in the normal bone remodeling and an imbalance between bone resorption and bone formation (Sakai et al., 1998; Miller et al., 2008 and Zhang et al., 2009). Epidemiologic studies on osteoporosis should consider oxidative stress (OXS), in addition to risk factors linked with lifestyles, hormonal changes and aging (KleereKoper, 1996 and Sanchez-Rodriguez et al., 2007).

Oxidative stress is a biochemical disequilibrium propitiated by excessive production of free radicals (FR) and reactive oxygen species (ROS), which provoke oxidative damage to biomolecules which can not counteracted by antioxidative systems (Foyer et al., 2008 and Minibayeva et al., 2009). This biochemical alteration has been linked with aging and more of 100 chronic-degenerative diseases, among which osteoporosis (Basue et al., 2001 and Isomura et al., 2004). One of the most damaging effects of ROS is lipid peroxidation, the end product of which is malondialdehyde (MDA) that also served as a measure of osteoclastic activity and (FR) production which overwhelms the natural antioxidant stress and thus leading to osteoporosis (Sheweita and Khoshhal, 2007). Oxidative stress that underlies physiologic organismal aging may be a pivotal pathogenetic mechanism of the age-related bone loss and strength (Almeida et al., 2007). The relationship between oxidative stress and osteoporosis was investigated in human (Ozgocmen et al., 2007; Sanchez-Rodriguez et al., 2007 and Altindag et al., 2008).
The ovariectomized model for osteoporosis should be curtailed due to its longer and more complicated experimental process. Utilizing the side effects of vitamin A of osteoporosis, an osteoporotic rat model can be made (Xu et al., 2006; Lei et al., 2007 and Wei et al., 2007). Oxidative stress generated by retinol in vitro was demonstrated (Gimeno et al., 2004 and Conte da Frota et al., 2006), but little available data about the oxidative stress of vitamin A in vivo and the effect of different time periods on this osteoporotic model. So, the present work aims to evaluate an osteoporotic rat model by administration of vitamin A for one, two and three weeks. Again, to evaluate the oxidative stress as a risk factor of vitamin A induced osteoporosis.

MATERIAL AND METHODS

**Animals:**
Male albino rats, *Rattus norvegicus*, (100-120 g) were used in all experiments. Animals were grouped and housed in polyacrylic cages (six animals per cage). Animals were fed on standard pellet and given water *ad libitum*. Rats were acclimatized to laboratory conditions for 7 days before commencement of the experiment.

**Vitamin A treatment:**
Retinoic acid tablets (A. viton) obtained from KAHIRA PHARM & CHEM. IND. CO. CAIRO, EGYPT, which was prepared as a suspension in distilled water and then stored under light-protection.

**Experimental Design:**
Twenty eight rats were separated into normal control group of 7 rats (group 1) and osteoporotic groups (21 rats, 7 rats / group). The osteoporosis was induced in rats by intragastric administration of vitamin A at the daily dose of 80 mg/kg b.wt (Wei et al., 2007) for 7 consecutive days (group 2), 15 consecutive days (group 3), and 21 consecutive days (group 4). At the end of each experiment rats were sacrificed, blood, liver and femora were collected for different biochemical analysis.

**Collection of Blood:**
Blood sample was immediately collected from each animal of the control and treated groups, in clean centrifuge tubes and centrifuged at 3000 rpm. for 20 minutes. Serum, stored at -20°C until used for biochemical assays. The appropriate kits (Stanbio-Laboratory) were used for the determination of serum aminotransferase activities ALAT & ASAT according to Reitman and Frankel (1957), alkaline phosphatase (ALP) activity (Young et al., 1975), calcium level (Harrison and Harrison, 1955) and phosphorus content (Fiske & Subbarow, 1925).

**Tissue analysis:**
Liver tissues were homogenized in 0.15 M Tris-HCl buffer (pH 7.4) and centrifuged at 9000 rpm. for 15 min. at 4°C. The supernatant obtained was used for malondialdehyde (MDA), catalase (CAT) and glutathione reduced (GSH) determination according to the methods described by Ohkawa et al. (1979), Aebi (1984) and Aykac et al. (1985) respectively using test kit (Bio-Diagnostic). At once femora were freed of soft tissue using small scissors, tweezers and cotton gauze. The left femora were minced and homogenized (100 mg/ml) at 4°C in 0.15 M Tris-HCl buffer (pH 7.4) and centrifuged at 3000 rpm for 10 min. The supernatant was collected and used for ALP activity and the oxidative markers (MDA, CAT & GSH) determination.

The right femora were used for the determination of bone calcium and phosphorus by ashing, bone mineral density and the bone histomorphometric examination.

**Bone Calcium and Phosphorus:**
The right femora were dehydrated and then carbonized by burning to ash in a furnace at 800°C for 8 hrs. The obtained ash was dissolved in 6 mol/l HCl to determine the bone calcium and phosphorus content spectrophotometrically.

**Bone Mineral Density (BMD):**
The total length and width (midshaft) of right femora were measured. The bone mineral density (BMD, g/cm²) was measured by dual-energy X-ray absorptiometry (DEXA) by using the small animal scan mode (Bastoureau et al., 1995).
Histological Preparation of Bone:
The right femur of each mouse was separated and fixed in 10% neutral buffered formalin (NBF), then decalcified in decalcifying solution for 30 days (mixed decalcifying solution was exchanges once a day for 30 days). After that, embedded in paraffin, sectioned and stained with hematoxylin and eosin stain.

Bone Histomorphometry:
In each prepared histological sample of right side of femur, percentage of trabecular area (% Tb. Ar), trabecular thickness (Tb. Th) and trabecular number (Tb. N) were measured using automated image analyser computer system (software Leica Qwin 500) under magnification of 100 microscopy in the uniform area of the right femur (growth plate regions were excluded).

RESULTS AND DISCUSSION

Effect of Vitamin A Administration on Serum Parameters:
The serum activities of ASAT and ALP were significantly (P<0.05) increased after seven days of vitamin A administration, as compared to the corresponding ones of the control rats with percentage changes of 10.73 and 29.92, respectively (Table 1 and Fig. 1). Daily administration of vitamin A for 15 days significantly increased (P<0.05) ALAT, ASAT and ALP levels of the treated rats. Meanwhile, twenty one days administration of vitamin A caused significant increase (P<0.05) in the activities of serum ALAT and ALP of rats with percentage difference of 28.33 and 61.13, respectively, as compared to the corresponding ones of the control group. Serum calcium and phosphorus contents of rats orally administered vitamin A were significantly decreased (P<0.05) after all the experimental periods, as compared to the corresponding ones of the control rats (Table 2 & Fig. 1).

Oxidative Stress in Liver:
The localization of radical formation resulting in lipid peroxidation, measured as MDA in rat liver supernatant as shown in table (3). Liver MDA was significantly (P<0.05) increased following vitamin A administration for 7, 15 and 21 days. However, a significant decrease (P<0.05) was noticed in the liver CAT activity and GSH level after all the experimental periods of vitamin A administration. Maximum decrease was recorded subsequent to 21 days of vitamin A administration for CAT activity and GSH level, as compared to the corresponding ones of control rats (Table 3 & Fig. 2).

Bone Oxidative Markers and ALP Activity:
MDA, a marker of lipid oxidative damage in bone supernatant, was significantly increased (P<0.05) after 7, 15 and 21 days of vitamin A administration, as compared to the control group (Table 3). Maximum increase was recorded after 15 days of vitamin A administration. However, significant decrease (P<0.05) was noticed in the bone CAT activity and bone GSH level after all the experimental periods of vitamin A administration, as compared to the corresponding ones of the control rats (Table 3 & Fig. 3). The percentage difference for both bone CAT and GSH were maximum after 15 days of vitamin A administration (-54.23 and -55.73, respectively). Bone ALP activity of rat daily administered vitamin A for 7, 15 and 21 days was significantly decreased (P<0.05), as compared to the control group (Table 1 & Fig. 3).

Effect of Vitamin A Administration on Bone, Calcium, Phosphorus and Mineral Density (BMD):
Vitamin A administration showed significant decrease (P<0.05) in bone calcium content after all the experimental periods, as compared to that of the control group (Table 2 &Fig. 4). This decrease was with maximum value (percentage difference, -29.68) after 21 days of vitamin A administration. However, bone phosphorus content was significantly decreased (P<0.05) only after 21 days of vitamin A administration, as compared to the control group (Table 2). Rats orally administered vitamin A daily for 7, 15 & 21 days had significant lower bone density of the right femora, as compared with the control ones (Table 2 & Fig. 4).

Histomorphometric Examination:
Morphological observations were quantifyed by histomorphometric analysis of longitudinal cross sections obtained from the right femur. A marked bone loss was noticed following vitamin A intake after the three experimental periods as compared to the control group (Table, 4). This bone loss was accompanied with a significant decrease (P<0.05) in Tb.Th and Tb.N (Table 4). This decrease starts from the first week till the third week. However, the % Tb.Ar was decreased significantly (P<0.05) following hypervitaminosis A for 15 and 21 consecutive days only (Fig. 5).
Fig. 1: Percentage changes in the aminotransferases (ALAT) and (ASAT), alkaline phosphatase (ALP) and serum calcium and phosphorus following vitamin A administration in male rats for one, two and three weeks.

Fig. 2: Percentage changes in the oxidative stress markers in the liver (MDA), (CAT) and (GSH) following vitamin A administration for one, two and three weeks.
Fig. 3: Percentage changes in the oxidative stress markers in the bone (MDA), (CAT), (GSH) as well as alkaline phosphatase (ALP) following vitamin A administration for one, two and three weeks.

Fig. 4: Percentage changes in the bone mineral density (BMD) and calcium and phosphorus concentrations in the serum and bone following vitamin A administration for one, two and three weeks.

Table 1: Effect of vitamin A on some biochemical parameters of male rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum ALAT(U/ml)</th>
<th>Serum ASAT(U/ml)</th>
<th>Serum ALP(U/L)</th>
<th>Bone ALP(U/gm. Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59.76 ± 4.26</td>
<td>86.70 ± 5.70</td>
<td>151.57 ± 14.72</td>
<td>105.45 ± 13.07</td>
</tr>
<tr>
<td>1st week</td>
<td>57.50 ± 2.01</td>
<td>96.00 ± 1.68</td>
<td>196.92 ± 14.20</td>
<td>148.72 ± 12.33</td>
</tr>
<tr>
<td>% difference</td>
<td>-3.75</td>
<td>10.73</td>
<td>29.92</td>
<td>41.03</td>
</tr>
<tr>
<td>2nd week</td>
<td>85.75 ± 3.09</td>
<td>98.49 ± 2.31</td>
<td>254.81 ± 20.07</td>
<td>145.51 ± 9.70</td>
</tr>
<tr>
<td>% difference</td>
<td>13.60</td>
<td>68.11</td>
<td>37.99</td>
<td>141.25 ± 20.45</td>
</tr>
<tr>
<td>3rd week</td>
<td>76.69 ± 1.77</td>
<td>91.41 ± 2.29</td>
<td>244.23 ± 13.78</td>
<td>141.25 ± 20.45</td>
</tr>
<tr>
<td>% difference</td>
<td>28.33</td>
<td>5.43</td>
<td>61.13</td>
<td>33.99</td>
</tr>
</tbody>
</table>

All data are mean of six rats ± SEM

a: significant at p < 0.05 as compared to the control.
Fig. 5: Photomicrographs of trabecular bone (Tb) undecalcified section (100x magnification) shows trabecular bone in control (A), group 1 (B) group 2 (C) and group 3 (D).

Table 2: Effect of vitamin A on calcium and phosphorus in the serum and bone and on the bone mineral density of male rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum</th>
<th>Bone</th>
<th>BMD (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calcium (mmol/L)</td>
<td>Phosphorus (mmol/L)</td>
<td>Calcium (mmol/L)</td>
</tr>
<tr>
<td>Control</td>
<td>3.2 ± 0.12</td>
<td>2.65 ± 0.08</td>
<td>4.11 ± 0.09</td>
</tr>
<tr>
<td>1st week</td>
<td>2.42 ± 0.11*</td>
<td>1.92 ± 0.09*</td>
<td>3.43 ± 0.07*</td>
</tr>
<tr>
<td>% difference</td>
<td>-24.38</td>
<td>-27.55</td>
<td>-16.55</td>
</tr>
<tr>
<td>2nd week</td>
<td>2.36 ± 0.09*</td>
<td>1.85 ± 0.05*</td>
<td>3.08 ± 0.13*</td>
</tr>
<tr>
<td>% difference</td>
<td>-26.25</td>
<td>-30.18</td>
<td>-25.06</td>
</tr>
<tr>
<td>3rd week</td>
<td>2.29 ± 0.08*</td>
<td>1.84 ± 0.08*</td>
<td>2.89 ± 0.16*</td>
</tr>
<tr>
<td>% difference</td>
<td>-28.44</td>
<td>-30.56</td>
<td>-29.68</td>
</tr>
</tbody>
</table>

All data are mean of six rats ± SEM
*a: significant at p < 0.05 as compared to the control.

Table 3: Effect of vitamin A on malondialdehyde (MDA), Catalase (CAT) and glutathion reduced (GSH) in the liver and bone of adult male rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver</th>
<th>Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDA (mmol/mg protein)</td>
<td>CAT (mU/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>7.15 ± 0.21</td>
<td>242.08 ± 4.32</td>
</tr>
<tr>
<td>1st week</td>
<td>10.66 ± 0.37*</td>
<td>142.74 ± 6.01*</td>
</tr>
<tr>
<td>% difference</td>
<td>49.1</td>
<td>-41.0</td>
</tr>
<tr>
<td>2nd week</td>
<td>9.92 ± 0.73*</td>
<td>134.89 ± 3.53*</td>
</tr>
<tr>
<td>% difference</td>
<td>38.74</td>
<td>44.28</td>
</tr>
<tr>
<td>3rd week</td>
<td>12.77 ± 0.95*</td>
<td>9.91 ± 4.15*</td>
</tr>
<tr>
<td>% difference</td>
<td>78.60</td>
<td>-59.06</td>
</tr>
</tbody>
</table>

All data are mean of six rats ± SEM
*a: significant at p < 0.05 as compared to the control.
Table 4: Bone histomorphometric parameters in control and vitamin A groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Tb. Ar.</th>
<th>Tb. Th. (μm)</th>
<th>Tb. N.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.22 ± 1.91</td>
<td>151.54 ± 23.56</td>
<td>18 ± 0.01</td>
</tr>
<tr>
<td>1st week</td>
<td>13.22 ± 2.40</td>
<td>119.07 ± 20.24*</td>
<td>16 ± 0.03*</td>
</tr>
<tr>
<td>2nd week</td>
<td>8.2 ± 0.72*</td>
<td>69.22 ± 13.78&quot;</td>
<td>15 ± 0.02&quot;</td>
</tr>
<tr>
<td>3rd week</td>
<td>6.83 ± 0.54&quot;</td>
<td>64.68 ± 9.63*</td>
<td>6 ± 0.04*</td>
</tr>
</tbody>
</table>

All data are mean of six rats ± SE
a: significant at p < 0.05 as compared to the control.

Discussion:

Vitamin A toxicity occurs when the capacity of the liver to store the vitamin is exceeded and the retinol-binding protein in the blood is overwhelmed (Nollevaux et al., 2006). Hepatic storage of vitamin A will continue until a pathologic liver condition develops (Hathcock et al., 1990 and Penniston and Tanumihardjo, 2006). The present study revealed a significant increase in the activities of the serum ALAT and ASAT in accordance with Ha et al. (2006), which may be due to the altered permeability of the hepatocyte membrane which causes leakage of the enzymes from the hepatic cells into circulation (Drotman and Lawhorn, 1978). Additionally, the hypervitaminosis with vitamin A induced rise of both serum and bone alkaline phosphatase activity as a biochemical marker of bone turnover, suggesting osteoporotic condition (Xu et al., 2006; Lei et al., 2007 and Wei et al., 2007). The oscillation of the ALP level in vitamin A-induced osteoporosis model of rat was associated with hyperfunctional bone resorption that vicariously enhanced the bone transformation as reported by Wei et al. (2007).

Hypervitaminosis A associated bone loss involves several mechanisms among of which the direct effects of vitamin A on bone cells as it is suppress osteoblast activity and stimulate osteoclast formation (Scheven and Hamilton, 1990 and Kindmark et al., 1995). cause bone loss through reduction of radial bone growth (Kneissel et al., 2005). Conte da Frota et al. (2006) provided evidence for a free radical generation by retinoic acid. Oxidative stress may be another mechanism through which vitamin A induces osteoporosis (Gimeno et al., 2004 and Conte da Frota et al., 2006). Retinoic acid was found to decrease estrogen level in blood (Wu et al., 1996). In addition, recent evidences implicate reactive oxygen species (ROS) in estrogen deficiency induced bone loss (Muthusami et al., 2005).

Lipid peroxidation is one of the major characteristics that can be included as an oxidative damage marker. Malondialdehyde (MDA) is served as a measure of osteoclastic activity and free radicals production (Shewcita and Khoshhal, 2007). Level of MDA in the present study was significantly increased in both liver and bone tissues followed the three experimental periods indicating an oxidative stress. In agreement with the previous result, Sheweita and Khoshhal, (2007) reported that when bone fracture occurs, a remarkable yield of free radicals is generated by the damaged tissues. Enhanced osteoclastic activity observed in hypervitaminosis A (Scheven and Hamilton, 1990 and Kindmark et al., 1995) may responsible for increased production of reactive oxygen species in the form of superoxide, which is evident by increased levels of MDA.

GSH is the frontline of the antioxidant system, but it seems not to be sufficient to prevent the cytotoxicity of ROS. In accordance with this finding, in our study we observed a significant decrease in the levels of GSH in both liver and bone tissues as compared to control group and following all experimental periods. Gimeno et al. (2004) reported that the oxidized glutathione (GSSG)/reduced glutathione (GSH) ratio and MDA production indicate that retinol, at concentrations greater than the physiological limit, induces oxidative stress. CAT is a hemoprotein which catalysis the reduction of hydrogen perixode and protects the tissue from highly reactive hydroxyl radicals (Rajasekaran et al., 2005). Since MDA has got high reactivity towards amino groups, it inhibits the synthesis of nucleic acids and proteins and also deactivates the enzymes (Bird and Draper, 1980). Thus, the decrease in liver and femur antioxidant markers observed after high dose of vitamin A treatment in the present study may be due to heightened lipid peroxidation, suggesting that oxidative stress induced in these osteoporotic models probably is the major factor behind the bone loss in these animals. These observations are consistent with osteoporosis recorded in other animal models subjected to oxidative stress (Basu et al., 2001; de Boer et al., 2002; Tyner et al., 2002 and Muthusami et al., 2005).

Femur is the longest bone provides mechanical support, protects bone marrow, stromal cells and participates in metabolic homeostasis (Ramajayam et al., 2007). Bone mineral density (BMD) has been regarded as a valuable index to test the changes of bone quality in clinics especially to human and generally decreased in osteoporosis animals regardless of the cause. It has been found that, there is a negative correlation between the OXS and the reduced bone density (Basu et al., 2001; Ozgocmen et al., 2007 and Altindag et al., 2008). In the current study BMD decreased with the same constant level (-37.5 percentage difference) from the 1st week till the 3rd week of treatment. This constant decrease may confirm that hypervitaminosis A can induces an osteoporosis rat model after only 7-days of treatment. Results of structural bone histomorphometric analysis revealed that vitamin A exerted negative effects on structural trabecular bone parameters by reducing Tb. Ar. and Tb. Th. The recorded bone loss we noted may be due to an increase in bone resorption secondary
to an increase in osteoclasts, as has been demonstrated both in cultured bone (Kindmark et al., 1995 and Kocijancic et al., 1995) and histologically (Dhem and Goret, 1984). Thus, the rate of bone formation is insufficient to keep up with resorption.

It is well known that calcium, phosphorus and estradiol are widely accepted phenotype markers of the bone formation (Evans et al., 1990). In this study, hypervitaminosis A markedly decreased the concentrations of Ca and phosphorus in both serum and bone. This decrease starts after 1st week of vitamin A treatment and continuous till the 3rd week. This decrease may be due to the antagonize effect of vitamin A on vitamin D (Johansson and Melhus, 2001).

In conclusion, results of oxidative stress observed following one week of vitamin A treatment, the constant decrease in BMD from the 1st week till the 3rd week and the results of structural bone histomorphometric analysis may reflect that hypervitaminosis A-induced osteoporotic model after only 7 days rather than 15 days.

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