Role of Alendronate and Alfacalcidol in Protection Against Steroid Induced Osteoporosis in Adult Male Albino Rats

HUSSEIN M. FAHMI, M.D.*; ESAM SALAH KAMEL, M.D.**; SALWA M. OUIES, M.D.** and ABEER F. ABD EL-NAEEM, M.D.**
The Department of Anatomy & Embryology, Faculty of Medicine, El-Azher University* and The Department of Human Anatomy & Embryology, Faculty of Medicine, Sohag University**

Abstract

Introduction: Glucocorticoid-Induced Osteoporosis (GIO) is the most prevalent form of secondary osteoporosis, during therapy, osteoporotic fractures are one of the most devastating conditions, affecting 30-50% of patients. Alendronate is the bisphosphonates of choice for postmenopausal and glucocorticoid induced osteoporosis. It has been also recommended for the prevention of bone loss in perimenopausal women. Alfacalcidol has been widely used since 1981 as a prodrug for calcitriol in the treatment of hypocalcemia, chronic renal failure, hypoparathyroidism and osteoporosis.

Aim of the Work: To investigate the effects of the combined effect of alendronate and alfacalcidol on steroid induced osteoporosis in rats, through histological and electron microscope examination and morphometric study.

Material and Methods: 40 adult male rats were divided into 4 groups Group 1: Control group; Group 2: Given oral prednisolone, 1mg/kg for 12 weeks; Group 3: Given oral prednisolone with administration of alendronate (0.2mg/kg daily, orally) for 12 weeks; Group 4: Given prednisolone with administration of alendronate + alfacalcidol (0.0375 µg/kg, orally) daily for 12 weeks. Animals were sacrificed, the head of tibia was taken and proceeded for light and electron microscopic examination.

Results: By light microscope rats treated with steroids only showed signs of osteoporosis in the form of thin trabeculae and fragmentations with wide marrow spaces.

After treatment with alendronates the rats showed signs of improvement; trabecular bone appeared more thick than group 2 with less widening of marrow spaces. The addition of alfacalcidol to the previous drugs showed that trabeculae was near normal with no fractures. By SEM rats treated with steroids only showed thinning, tapering, and breakage of trabeculae with irregular arrangement of the collagen fibrils. After treatment with alendronates the trabeculae increased in volume and number than group 2 with regular arrangement of the collagen fibrils. The addition of alfacalcidol to the previous drugs showed normal appearance of bone trabeculae with regular arrangement of the collagen fibers.

Conclusions: Alendronates are usefull in treatment of steroid induced osteoporosis and the addition of alfacalcidol gives synergistic effect to it.

Key Words: A lendronates – Steroid – Alfacalcidol – Bone.

Introduction

OSTEOPOROSIS is a systemic skeletal disease associated with a low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in the bone fragility and fracture risk [1]. Patients with a Bone Mineral Density (BMD) <70% of young adult mean or 70%-80% with a history of osteoporotic fractures were diagnosed as having osteoporosis [2].

Glucocorticoids (prednisolone) are important therapeutic agents that have been used for their potent anti-inflammatory and immunosuppressive properties for over 50 years [3,4].

Glucocorticoid-Induced Osteoporosis (GIO) is the most prevalent form of secondary osteoporosis [5]. During therapy, osteoporotic fracture is one of the most devastating conditions, affecting 30-50% of patients [4].

The skeletal effects of glucocorticoids include both direct and indirect actions on bone that result in an early, transient increase in bone resorption accompanied by a decrease in bone formation, which is maintained for the duration of glucocorticoid therapy [6].

Bisphosphonates is a group of inorganic pyrophosphates analogues that suppress bone resorption by inducing an osteoclast inactivation, being frequently used for the management of diseases af-
fecting bone metabolism, bone metastases and bone tumors [7,8]. Alendronate is one of the best and most extensively studied bisphosphonates in the treatment of osteoporosis [9].

Alendronate is the drug of choice for postmenopausal and glucocorticoid induced osteoporosis. It has been also recommended for the prevention of bone loss in perimenopausal women. Currently, it has been also viewed as a treatment option in osteoporotic males. Several clinical trials have been stated the significant role of alendronate in the management of osteoporosis [10].

Alfacalcidol (1α-hydroxyvitamin D3) has been widely used since 1981 as a prodrug for calcitriol (1, 25-dihydroxyvitamin D3) in the treatment of hypocalcemia, chronic renal failure, hypoparathyroidism and osteoporosis [11].

Treatment with Vitamin D is a nutritional substitute, while the application of alfacalcidol is an active hormonal therapy. Nevertheless, a majority of physicians still prescribe plain Vitamin D plus calcium as a first-step prevention or even as therapy for glucocorticoid induced osteoporosis [12].

Alfacalcidol prevent the age related cancellous bone loss and cortical bone changes by increasing cancellous bone density and improves trabecular structure resulting in an increase of bone strength [13].

During the course of the alfacalcidol treatment period, the combination of maintained bone formation and decrease bone resorption on cancellous bone surfaces of aged rats produce a positive bone balance between bone formation and resorption resulting in thickening of trabecular bone and a net bone gain in the aged animals [13,14].

Aim of the work:

To investigate the combined effect of alendronate and alfacalcidol on steroid induced osteoporosis in rats, through histological and electron microscopic examination and morphometric study.

Material and Methods

Animals:

Forty adult male albino rats weighing 150-200 gm/each were used in the present study. All animals were housed under the same conditions and allowed food and water. Rats were randomly divided into four equal groups.

- Group 1: Control group; given 1ml distilled water.
- Group 2: Given prednisolone (product of Kahira-Egypt, in the form of trade name of prednilone) 0.1mg/kg [15].
- Group 3: Given prednisolone& alendronate (product of Global Napharmacititecal-Egypt under authority of Merk-co. Inc. USA in the form of tablets 70mg under trade name of Fosamax) 0.2mg/kg [16].
- Group 4: Given prednisolone, alendronate & alfacalcidol (product of Leo pharmaceutical of Denmark in the form of tablets 0.5 μg under trade name of one alpha) 0.0375 μg/kg [16].

Each dose was dissolved in 1ml distilled water and orally administered daily for 3 months. After 24hrs of the last dose, the animals were sacrificed by cervical dislocation, the head of the tibia was taken and subjected to light and scanning electron microscopic studies.

For light microscopic study: At first, the head of the tibia was decalcified by daily exchange in EDTA (Ethylene Diamine Tetra-Acetic acid) then specimens were fixed in 10% formalin, processed and embedded in paraffin. Serial sections (5 microns) were prepared and subjected to hematoxylin and eosin stains.

For Scanning Electron Microscopic study (SEM): Sections of the head of the tibia were washed with normal saline, rinsed with cocodylate buffer and placed in 2.5% glutaraldehyde. Following fixation, the specimens were washed several times with cold cocodylate buffer and post-fixed in 1% osmium tetroxide. They were dehydrated in a graded ethanol series, exposed to liquid CO2 in a drying apparatus and coated with a thin layer of gold (10-15um) deposited over the surface in vacuum evaporator. The specimens were examined with a Jeol-JSM-5400 LV scanning electron microscope in Assiut University.

Morphometric methods and statistical studies:
The following parameters were quantitated:

- The thickness of bone trabeculae; trabecular thickness was defined as thickness of the trabecular bone between two points away from the branching of the trabeculae from hematoxylin and eosin-stained sections at magnification of 100.
- The distance between the trabeculae (bone marrow spaces) measured from hematoxylin and eosin-stained sections at magnification of 100.
In each section five measurements were obtained from five randomly chosen fields using image analysis system (digimizer version 3.7, 2005-2010) software in the Anatomy Department at Sohag University.

Variables were represented by mean ± SD (mean ± standard deviation). The data were statistically analysed using the one way ANOVA test. A probability value of \( p < 0.05 \) was considered significant and \( p < 0.01 \) was considered highly significant.

**Results**

**Group 1 (control group):**

**Histological study:**

Examination of hematoxylin and eosin-stained sections from the control group showed normal architecture of the cancellous bone; the tissue arranged as trabeculae which appeared as numerous interconnecting bone marrow spaces of various sizes with bone marrow cells in-between Fig. (1A).

Osteocytes were seen as basophilic cells inside their lacunae in the bone lamellae. Osteoblasts appeared with their large size cuboidal or polygonal in shape, with eccentric nuclei, their cytoplasm was heavily basophilic and they were aggregated into a single layer of cells lining the trabeculae Fig. (1B).

**Scanning Electron Microscopical (SEM) examination:**

Examination of the head of the tibia with SEM showed cancellous bone which consisted of trabeculae or lamellae having rod shape or plate like shape with the bone marrow spaces inbetween (honey comb appearance) Figs. (1C,D). The collagen fibers in the trabeculae were seen with regular arrangement Figs. (1D,E).

**Group 2 (prednisolone group):**

**Histological study:**

By light microscope bone shows the picture of osteoporosis; the bone lost its normal architecture, trabeculae were thin, reduced in number and sometimes fragmented and fractured with wide marrow spaces Figs. (2A,B). Almost no osteoblastic cover was observed with few scattered cells which appeared inactive with flat nuclei.

**Scanning electron microscope examination:**

The bone was composed of either plate-like or rod-like trabeculae which had been resorbed to become interrupted and form various stump structures Fig. (2D). Changes such as thinning, tapering, breakage, and perforation made the trabeculae lose its integrity. These changes contributed to an obviously increasing separation and porosity of inter-trabeculae Fig. (2E).

The collagen fibrils on the surface of the trabeculae presented irregular arrangement and breakage. Figs. (2E,F).

**Group 3 (prednisolone group treated with Alendronate):**

**Histological study:** The bone of rats treated by prednisolone and alendronate showed many changes. The bone trabeculae, osteocytes and osteoblasts showed improvement. Trabecular bone appeared more thick than group 2. The bone lamellae were mainly arranged in regular pattern. The matrix appeared homogenous with less widening of marrow spaces Fig. (3A). Osteocytes appeared active with large nuclei located in wide lacunae. The osteoblasts were seen covering the trabeculae, they appeared cuboidal in shape with active nuclei Fig.

**Scanning electron microscope examination:**

The trabeculae increased in volume and number than Group 2, with still fractures are seen Fig. (3C). The bone marrow cavities were less wide than Group 2 with less porosity Fig. (3D).

Collagen fibers appeared more regular in arrangement and less fragmented than the rats treated with steroids only Figs. (3D,E).

**Group 4 (prednisolone group treated with alendronate + Alfacalcidol):**

**Histological study:** Rats treated with prednisolone, alendronates and alfacalcidol showed that the bone was near to normal picture: Trabeculae were thick with no fractures or fragmentations were seen Fig. (4A). Osteocytes were seen with large nucleus, filling the lacunae. Osteoblasts were lining the trabeculae with numerous amounts, and appeared large size with cuboidal nucleus Fig. (4B).

**Scanning electron microscope examination:**

Sections of the head of tibia in this group showed better changes. The bone trabeculae appeared similar in thickness and number to the control group. The bone marrow spaces decreased in size, with the least fragmentations Fig. (4C).

The collagen fibers were regularly arranged with no breakage between them Figs. (4D,E).

**Morphometric results:**

Trabecular thickness (Table 1), Histogram (1) the mean trabecular bone thickness in Group 2 and
Group 3 showed a highly significant ($p<0.00$) decreased compared with the control group. The mean trabecular bone thickness in Group 4 showed non-significant ($p<0.55$) change compared with the control group.

Trabecular separation: (Table 1), Histogram (2) the mean trabecular separation (bone marrow spaces) in Groups 2, 3 & 4 showed a highly significant ($p<0.00$) increase when compared with the control group ($p<0.00$).

Fig. (1A): A photomicrograph of a transverse section of a head of the tibia of control group showing a network trabeculae of cancellous bone (TB). Bone marrow spaces are seen between the trabeculae (BM). (Group I: H & E X100).

Fig. (1B): A photomicrograph of a transverse section of a head of the tibia of control group showing the osteocytes inside their lacunae (OC). The osteoblasts are seen as cuboidal cells covering the surface of the lamellae (OB) (Group I: H & E X400).

Fig. (1C): An electro micrograph of a head of the tibia of control group showing the trabeculae appear with rod shape (TB). The bone marrow spaces are seen between the trabeculae (BM). (Group I: SEM X200).

Fig. (1D): An electro micrograph of a head of the tibia of control group showing one of the trabeculae (TB) having plate shape with normal arrangement of collagen fibers (arrows) (Group I: SEM X500).

Fig. (1E): An electro micrograph of a head of the tibia of control group showing the collagen fibers with their normal arrangement (arrows). (Group I: SEM X1.500).
Fig. (2A): A photomicrograph of a section of a head of tibia of a rat treated with prednisolone showing thinning of the trabeculae (TB) with fractures inside them (arrows). The bone marrow spaces are seen wide between the trabeculae (BM) and having lipidemia (L) (Group II: H & E X100).

Fig. (2B): A photomicrograph of a section of a head of tibia of a rat treated with prednisolone. The trabeculae (TB) are seen fragmented and discontinuous (arrows), with wide bone marrow spaces (BM) having lipidemia (L) (Group II: H & E X100).

Fig. (2C): A photomicrograph of a section of a head of tibia of rat treated with prednisolone. Few osteocytes (OC) and osteoblasts (OB) are seen (Group II: H & E X400).

Fig. (2D): An electron micrograph of a section of head of tibia of a rat treated with prednisolone. The trabeculae show breakage, and perforations (arrows). There are wide BM spaces (BM). (Group II: SEM X200).

Fig. (2E): An electron micrograph of a section of a head of tibia of a rat treated with prednisolone. The trabeculae (TB) appear thin and irregular, with detached parts (arrows) (Group II: SEM X500).

Fig. (2F): An electron micrograph of a magnified part of previous picture showing collagen fibers in TB which appear irregular and interrupted (arrows) (Group II: SEM X1,500).
Role of Alendronate & Alfacalcidol in Protection Against Steroid Induced Osteoporosis

Fig. (3A): A photomicrograph of a section of a head of tibia of a rat treated with alendronates + prednisolone; intact thick trabeculae are seen (TB) around the BM spaces. (Group III: H & E X100).

Fig. (3B): A photomicrograph of a section of a head of tibia of a rat treated with alendronates + prednisolone; the osteocytes are numerous with large nuclei located in wide lacunae (OC). The osteoblasts are seen also cuboidal in shape (OB). (Group III: H & E X400).

Fig. (3C): An electro micrograph of a section of a head of tibia of a rat treated with alendronates + prednisolone: The trabeculae appear thick, regular, more or less intact (TB). Still fractures are seen (arrows). BM spaces appear narrower than group 2 (BM). (Group III: SEM X200).

Fig. (3D): An electro micrograph of a section of a head of tibia of a rat treated with alendronates + prednisolone: The trabeculae appear thick (TB). With Some fractures are still seen (arrows). Bone marrow (BM) spaces are seen intact and filled with cells (Group III: SEM X500).

Fig. (3E): An electro micrograph of a magnified part of previous picture showing regular collagen fibers (arrows). Still areas of interruption are seen (Stars) (Group III: SEM X1.500).
Fig. (4A): A photomicrograph of a section of a head of tibia of a rat treated with prednisolone, alendronates & alfacalcidol: The trabecular appear thick and regular (TB). The BM spaces are narrow and filled with cells (BM). (Group IV: H & E X100).

Fig. (4B): Magnified part of the previous picture showing numerous osteocytes with large nucleus, filling the lacunae (OC). Osteoblasts are seen lining the trabeculae (OB). (Group IV: H & E X400).

Fig. (4C): An electro micrograph of a section of a head of tibia of a rat treated with one alpha + alendronates + prednisolone: The bone trabeculae appear thick and intact (TB) surrounding the bone marrow spaces (BM). (Group IV: SEM X200).

Fig. (4D): An electro micrograph of magnified part of the previous picture: Trabeculae (TB) appear thick and with regular borders (Group IV: SEM X500).

Fig. (4E): An electro micrograph of a section of a head of tibia of a rat treated with one alpha + alendronates + prednisolone: The collagen fibers are seen more regular in arrangement with no fragmentations (arrows). (Group IV: SEM X1.500)

Table (1): Comparison between different groups regarding bone thickness and bone marrow spaces (Trabecular separation).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Steroid</th>
<th>Alendronate</th>
<th>Alendronate + Alfacalcidol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean trabecular thickness</td>
<td>81.82±2.19</td>
<td>55.62±4.3 **</td>
<td>66.34±3.58 **</td>
<td>81.35±2.60</td>
</tr>
<tr>
<td>Mean trabecular separation</td>
<td>133.11±2.52</td>
<td>202.81±2.79 **</td>
<td>107.21±11.84**</td>
<td>108.15±.52 **</td>
</tr>
</tbody>
</table>

**: Highly significant in comparison to the control group.
Discussion

The present study evaluated the effects of alendronate in glucocorticoid treated rats and the additive effect of alfacalcidol on it. The dose of prednisolone 1 mg/kg body weight was used as standard treatment for Giant Cell Arteritis (GCA) [17]. The duration of treatment was 3 months as all patients on glucocorticoid are threatened with osteoporosis if put on a daily dose of prednisolone at least 3 months [18].

The head of tibia was used as an example of cancellous bone as the skeletal effects of glucocorticoids are observed mainly in regions with a high content of trabecular bone [19].

In the present study the rats treated with steroids only showed pictures of osteoporosis. With light microscope the bone showed many degenerative changes in the bone trabeculae which appeared thin with breakage in them. The BM spaces appeared more wide with replacement of the BM cells with fat lobules (Lipidemia). The osteoblasts became inactive with fusiform nucleus; the lacunae of osteocytes appeared empty from cells.

These results were in acceptance with a study showed that in animals with ovarectomy induced osteoporosis, bone marrow spaces were occupied with fat cells with decrease in the number of osteoblasts [20]. Glucocorticoid treatment induced osteonecrosis in trabecular bone which appeared with empty lacunae and osteocyte ghosts [21].

In a study of prednisolone-treated rats the total trabecular bone volume was 22% lower, trabecular number was 4% lower and trabecular thickness was 7% lower in the prednisolone-treated animals compared with the controls [22].

Scanning electron microscope of a head of tibia of the bone treated with prednisolone showed thin trabeculae, the bone marrow spaces were wider and the collagen fibers appeared irregular and fragmented. These results were also showed in the femoral head of osteoporotic women where osteoporotic trabeculae appeared with thinning, tapering, breaking and perforating changes [23]. Glucocorticoids made cancellous bone volume lower than controls [24].

Glucocorticoids act mainly on calcium and bone metabolism by disturbing vitamin D metabolism. They reduce I, 25-dihydroxyvitamin D receptors in bone leading to inflammation induced osteoporosis [25] or they decreased intestinal calcium absorption [15].

Glucocorticoids (GCs) negatively affect bone through multiple pathways; proinflammatory cytokines induce bone resorption, reduce bone formation and induce muscle dissipation [26,27]. Glucocorticoids also cause osteoblastic dysfunction by shortening the period in which the osteoblasts work actively to form the bone matrix [28,29].

The concomitant administration of steroids and alendronate sappeared to protect the cancellous bone from the effects of glucocorticoids as seen by light microscopy. Trabecular bone appeared thick, the matrix appeared homogenous with less widening of marrow spaces, the osteoblasts appeared active with cuboidal nucleus and the osteocytes appeared with large nucleus in the lacunae.
In a study of alendronate effect on femoral bone under glucocorticoids therapy the submicroscopic structure got better changes in osteoblasts and in the matrix; the osteoblasts appeared normal, the matrix appeared homogenous and the bone marrow spaces were less wide compared to the control [15].

The results of the electron microscopy confirmed the light microscopic findings and showed that the trabeculae increased in thickness, the bone marrow spaces were less wide and the collagen fibers were more regular than the steroid group. Alendronates effectively reduce the fracture risk in postmenopausal women at the highest risk because of advanced age or severe osteoporosis [30].

Study of the effect of the different subgroups of bisphosphonates on postmenopausal women revealed that patients on alendronates treatment appear better than those on other subgroups of bisphosphonates [31].

On the other hand patients who received intravenous bisphosphonates osteonecrosis of jaw had been occurred [32,33].

Bisphosphonates are the first drugs of choice in all the patients with increased risk for osteoporosis [29]. Alendronate causes significant increase in the number of osteoblasts [34], enhances proliferation of bone marrow stromal cells and initiates osteoblastic differentiation [35], prolongs the life span of osteoblasts and osteocytes [28] and induces apoptosis of osteoclasts [36-38].

Available evidence suggested that osteoclasts on the trabecular surface may be more responsive to bisphosphonates than those on the endocortical surface [39]. Thus, the response of the bone mineral density and bone strength to bisphosphonates might be more significant in the trabecular bone than in the cortical bone which is supported by the results reported from sciatic neurectomized rats [2].

The addition of alfalcacidol to the previous drugs showed the best results; the thickness of trabeculae increased; the number of osteocytes increased which appeared filling the lacunae and having large nucleus. The osteoblasts also appeared larger in size with cuboid nucleus. Bone volume of the rats treated with 0.1 and 0.2mg/kg/d of alfalcacidol was increased by 31% and 45%, respectively in aged male rats [13].

The results of the electron microscopy confirmed the improvement. The trabeculae appeared thick with narrow bone marrow spaces. The collagen fibers appeared regular with no breaks. A study in the lumbar spine reported a better effect of combined administration of alendronates and alfalcacidol on the cancellous bone mass compared with that of single administration of either agent alone in ovariectomized rats [40].

The combined treatment of alendronate and alfalcacidol showed the maximum gains in bone mineral density at lumbar spine and femoral neck in comparison to other treatments [41]. Others suggest that there was no indication in glucocorticoid treated patients for any combination of two antiresorptive agents, as alendronate alone was enough [15].

Recent data tends to support the use of Vitamin D analogs with some bisphosphonates in patients with severe bone loss and glucocorticoid induced osteoporosis [25,30,42].

From the previous results it can be concluded that:
- Steroids cause osteoporosis in bone.
- Alendronates are usefull in treatment of steroid induced osteoporosis.
- The addition of alfalcacidol gives synergistic effect to it.

Recommendation:
It is recommended to add alfalcacidol to alendronate in treatment of steroid induced osteoporosis.

References
Role of Alendronate & Alfacalcidol in Protection Against Steroid Induced Osteoporosis


