Effect of concomitant administration of eicosapentaenoic acid and alendronate on bone changes due to methylprednisolone in rats

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Abstract: One of the main limitations of glucocorticoid therapy is its negative effects on bones. The aim of this study was to determine the effects of concurrent administration of eicosapentaenoic acid (EPA) and alendronate on bone changes induced by glucocorticoid treatment in rats. Thirty six male Wistar rats, who were 2.5 months of age, were divided equally into six groups and treated with normal saline (controls), 7 mg/kg methylprednisolone (MP), MP with 20 µg/kg alendronate, MP with alendronate and 80 mg/kg EPA, MP with alendronate and 160 mg/kg EPA, and MP with alendronate and 320 mg/kg EPA for a six-week period. At the end of the experiment, serum and urine samples were collected and the left tibia and femur were removed from each animal for histomorphometric studies.

There were no significant differences with regards to the levels of serum calcium, phosphorus, creatinine, osteocalcin, carboxy-terminal telopeptide of collagen type I (CTX), alkaline phosphatase, urinary calcium and creatinine, and the phosphorus:creatinine ratios among all groups. Epiphyseal and metaphyseal trabecular widths, and the area of epiphyseal bone and the entire femur in the MP group decreased significantly in comparison to the control group (p<0.001). There were no significant differences in the cortical parameters of the tibial bone between these groups. The groups treated with alendronate and alendronate with 80 mg/kg EPA had increased epiphyseal trabecular widths compared to the MP group (p<0.001) that were statistically similar in both groups. The groups treated with alendronate with 80 mg/kg EPA and alendronate with 320 mg/kg EPA had increased metaphyseal trabecular widths compared to the MP group (p<0.001). The groups with alendronate treatment alone and alendronate with 80 mg/kg EPA had significantly increased bone areas and tissue areas compared to the MP group (p<0.001). In conclusion, concomitant administration of EPA and alendronate may improve the known beneficial effects of alendronate on changes in the bone due to MP administration in rats.

Keywords: Eicosapentaenoic acid, methylprednisolone, alendronate, bone, rats

Introduction

Despite the widespread and diverse use of glucocorticoids (GC), their negative impact on bone mass is one of the most important limitations of this treatment. GC-induced osteoporosis (GIO) is the most frequent cause of secondary osteoporosis (Gregorio *et al.,* 2006). It has been demonstrated that prolonged exposure to GC at supraphysiological doses induces osteoporosis and is associated with an increased risk of fractures (Wang *et al.,* 2002). Although bisphosphonates, which include alendronate and risedronate, have

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proven to be efficacious in the prevention and treatment of GIO, diet and lifestyle changes that minimize bone loss are helpful to decrease the necessity for drug therapy in the management of osteoporosis (Gregorio *et al.,* 2006; Sun *et al.,* 2003). Long chain polyunsaturated fatty acids (LCPUFAs) and their metabolites, which have been extensively studied with regards to their cardioprotective role, also regulate bone metabolism and may have potential for the prevention and/or treatment of osteoporosis (Poulsen *et al.,* 2007). A diet that was enriched with eicosapentaenoic acid (EPA), which is a LCPUFA from the n-3 family, prevented decreases in bone weight and strength in an animal model of osteoporosis (Sakaguchi *et al.,* 1994).

The aim of this study was to clarify the putative positive effects of concurrent administration of EPA and alendronate, which is a common anti-resorptive drug, alendronate, on bone changes due to GC administration in rats with respect to histomorphometric, serum and urinary parameters. The secondary aim of this study was to compare these results with those from the administration of alendronate as a single agent in rats treated with methylprednisolone (MP)**.**

Materials and Methods
Animals
Thirty six male Wistar rats who were nine weeks of age with a body weight of 300 ± 36 g (mean $\pm$ standard deviation [SD]), were purchased from the Razi Serum and Vaccine Research Institute in Iran. After a week of adaptation, they were divided randomly into six experimental groups with six animals in each. Each group was treated for six weeks with the following protocols:

1- Control group: 0.9% sodium chloride (NaCl) once a week subcutaneously (sc).
2- MP group: methylprednisolone sodium succinate (MP; Merck, France), 7 mg/kg once a week (sc).
3- MP + alendronate sodium trihydrate (Arasto Pharmaceutical and Chemicals Inc., Iran) 20 µg/kg twice a week (sc).

4- MP + alendronate + EPA (S.L.A. Pharma AG, Switzerland) 80 mg/kg daily by oral gavage.
5- MP + alendronate + EPA 160 mg/kg daily by oral gavage.
6- MP + alendronate + EPA 320 mg/kg daily by oral gavage.

The doses, dosage intervals and duration of treatment with MP and alendronate were chosen according to the study performed previously by Wimalawansa *et al*., (1998).

During the experimental period, the animals were weighed on a weekly basis and maintained on a 12h/12h light-dark cycle at $20 \pm 20C$. They were allowed free access to tap water and food, which was provided by the Razi Serum and Vaccine Research Institute in Iran. All animals were treated ethically in compliance with the local regulations of the Faculty of Veterinary Medicine at the University of Tehran.

Sampling of blood and bones
On day 43, urine samples were collected from all animals. The rats were then anesthetized with chloroform and blood samples were obtained by cardiac puncture. Serum samples were harvested within one hour after blood sampling, and serum and urine samples were stored at $-20^{0}C$ until analysis. After the animals were sacrificed under deep anesthesia, the left tibia and femur were dissected from each rat for histomorphometric studies.

Preparation of specimens for histomorphometric study
After the attached soft tissues were removed, the left tibias and femurs were fixed in 4% formaldehyde solution and decalcified using the formic acid-sodium citrate method (Armed Forces Institute of Pathology, 1960). Transverse 5 µm cross-sections were made from the tibial bone in a perpendicular orientation to the long axis at the distal point that the fibula attaches to the tibia (4-5 sections in each animal). From the femoral bone, 5 µm longitudinal sections of the distal epiphysis and metaphysis were made in the median plane. All sections were stained using Masson's trichrome method (Armed Forces

Institute of Pathology, 1960).The epiphyseal and metaphyseal trabecular widths were measured in the longitudinal sections of the femur, as well as the epiphyseal trabecularbone area: total tissue area. The mean widths of the epiphyseal and metaphyseal trabeculae were calculated from the measurements of 10 epiphyseal or metaphyseal trabeculae. These were measured in the central region between the two cortices within 2-3 mms below (epipyseal trabeculae) or above (metaphyseal trabeculae) the epiphyseal growth plate. In the cross-section of the tibias, the cortical widths, marrow area and cortical area were determined.All histomorphometric parameters were measured using a digital photo microscope connected to a personal computer with Ziess Axio Vision LE software.

Determination of serum and urine parameters

Serum osteocalcin and carboxy-terminal telopeptide of collagen type I (CTX) were measured using Rat-MIDTM osteocalcin enzyme-linked immunosorbant assay (ELISA) and RatLapsTM ELISA kits (Nordic Bioscience Diagnostics A/S, Denmark), respectively. Serum alkaline phosphatase was assayed by a photometric method (Pars Azmun Company, Ltd., Iran).

The levels of serum and urine calcium, inorganic phosphorus (Pi) and creatinine were determined by the cresolphthalein complexone method, the photometric method and the Jaffe method, respectively. All the reagents for these methods were prepared by the Pars Azmun Company, Ltd., Iran.

Statistical analysis

All data were expressed as mean ± SD. Data comparisons among groups performed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical analyses were performed using a Sigma Stat 2 statistical package. A significance level of $p < 0.05$ was set for all comparisons.

Results
Body weight

During the first four weeks of the experiment,

there was no significant difference in body weight between all groups. At the end of the fifth and sixth week, the body weights of the animals in the group treated with MP, alendronate and 160 mg/kg EPA were 17% and 19% lower than the MP group, respectively.

Histomorphometric parameters

The epiphyseal trabecular width decreased significantly (40.87%) in the MP group compared to the controls ($p < 0.001$). Alendronate alone and alendronate with 80 mg/kg EPA significantly increased this parameter with respect to the MP group ($p < 0.001$). This increase was statistically similar in the groups treated with alendronate alone and alendronate with 80 mg/kg EPA. The metaphyseal trabecular width decreased significantly (11.62%) in the MP group compared to controls ($p < 0.001$). Alendronate treatment alone did not produce a significant increase in the metaphyseal trabecular width in comparison to the MP group. However, the increase was significant in comparison to the groups treated with alendronate and 80 mg/kg EPA and alendronate and 320 mg/kg EPA compared to the MP group ($p < 0.001$). Bone area: tissue area ratio significantly (39%) decreased in the MP group compared to the control group ($p < 0.001$). This parameter was significantly increased in the groups treated with alendronate alone and alendronate with 80 mg/kg EPA in comparison to the MP group ($p < 0.001$ for both groups).

MP did not significantly alter the cortical width of the tibial diaphysis in comparison to the value recorded in the control group, and there was no significant decrease in this parameter in either the groups treated with alendronate alone or with alendronate and EPA when compared to the control group.

There was no significant change in the ratio of marrow area:cortical area of the tibial diaphysis in any of the experimental groups in comparison to the control group. A summary of the histomorphometric data is shown in Table 1.

Table 1: Histomorphometric parameters presented as the mean ± SD; n = 6 for each group.

	G1	G2	G3	G4	G5	G6
Epiphyseal trabecular width(μm)	63.4 ± 2.3	#37.5 ± 2.12	*#52.6 ± 2.61	*#54.2 ± 2.19	#47.8 ± 2.38	#42.8 ± 2.18
Metaphyseal trabecular width(μm)	38 ± 1.93	#33.6 ± 1.45	35.3 ± 1.38	*38.6 ± 1.78	36.6 ± 1.75	*43.3 ± 2.03
Bone area: tissue area (%)	38 ± 2.5	#23 ± 1.7	*35 ± 4.5	*33 ± 5.7	#29 ± 4.3	#29 ± 2.5
Cortical width of the tibial diaphysis(μm)	337.6 ± 25.8	347.4 ± 26.5	350.5 ± 16.67	360.7 ± 20	378.4 ± 17.89	351 ± 14.7
marrow area: cortical area ratio of the tibial diaphysis	0.24 ± 0.025	0.22 ± 0.021	0.25 ± 0.013	0.21 ± 0.022	#0.18 ± 0.011	0.25 ± 0.004

G1: control; G2: methylprednisolone (MP); G3: MP + alendronate; G4: MP + alendronate + 80 mg/kg eicosapentaenoic acid (EPA); G5: MP + alendronate + 160 mg/kg EPA; G6: MP + alendronate + 320 mg/kg EPA; SD: standard deviation.
*Significant difference when compared to the MP group (p<0.05).
Significant difference when compared to the control group (p<0.05).

Table 2: Serum parameters presented as mean ± SD; n = 6 for each group.

	G1	G2	G3	G4	G5	G6
Osteocalcin (ng/ml)	75.4 ± 5.1	56.9 ± 5.37	66.8 ± 22.2	59.9 ± 10.3	50.2 ± 5.1	68.5 ± 25.9
Alkaline phosphatase (mg/dl)	306.5 ± 51.8	320.8 ± 60.3	293.2 ± 48.04	260.33 ± 58.2	261 ± 62.2	305 ± 61.6
CTX (ng/ml)	12.5 ± 2.4	11.4 ± 1.8	10.5 ± 0.6	11.3 ± 1.1	10.4 ± 2.4	11.1 ± 2.0
Creatinine (mg/dl)	0.68 ± 0.07	0.6 ± 0.06	0.68 ± 0.12	0.6 ± 0.07	0.59 ± 0.06	0.56 ± 0.09
Calcium (mg/dl)	10.48 ± 2.42	10.51 ± 2.06	10.16 ± 1.64	8.27 ± 1.46	8.51 ± 1.51	10.47 ± 0.88
Phosphorus (mg/dl)	7.22 ± 1.16	7.68 ± 1.5	7.26 ± 1.27	7.22 ± 0.78	7.1 ± 2.58	9.6 ± 1.73

G1: control; G2: methylprednisolone (MP); G3: MP + alendronate; G4: MP + alendronate + 80 mg/kg eicosapentaenoic acid (EPA); G5: MP + alendronate + 160 mg/kg EPA; G6: MP + alendronate + 320 mg/kg EPA. There was no significant difference in the levels of serum CTX, osteocalcin, alkaline phosphatase, calcium and phosphate between all groups.

Table 3: Urinary parameters presented as the mean ± SD; n = 6 for each group.

	G1	G2	G3	G4	G5	G6
Calcium: creatinine ratio	0.04 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
Phosphorus: creatinine ratio	1.65 ± 0.49	1.88 ± 0.59	1.63 ± 0.06	2.67 ± 0.06	2.13 ± 0.55	2.4 ± 0.57

G1: control; G2: methylprednisolone (MP); G3: MP + alendronate; G4: MP + alendronate + 80 mg/kg eicosapentaenoic acid (EPA); G5: MP + alendronate + 160 mg/kg EPA; G6: MP + alendronate + 320 mg/kg EPA. There was no significant difference in the ratio of urinary calcium:creatinine and phosphate:creatinine between all six groups.

Serum and urinary parameters

There were no significant differences between all of the groups with regards to the levels of serum calcium, phosphorus (Pi) , creatinine, or the biochemical markers of bone metabolism, including osteocalcin, CTX, and alkaline phosphatase (Table 2). Urinary calcium:creatinine and phosphorus (Pi):creatinine ratios were statistically similar between the experimental groups (Table 3).

Discussion

Although previous studies have reported positive effects of EPA on bone loss due to estrogen deficiency, its effect on GIO has not been clarified completely. The aim of the present study was to evaluate the putative positive effects of concurrent administration of alendronate with EPA (at three different doses) on detrimental bone changes due to GC administration in rats, compared to the effects of alendronate alone.

The key histological feature of GC-induced cancellous bone loss is a reduction in the trabecular thickness, which reflects suppressed bone formation (Manolagas *et al*., 1999). This is consistent with the results of our study, which demonstrated appreciable decreases in the epiphyseal and metaphyseal trabecular widths in the MP group compared to the controls.

Although the effect of alendronate in increasing the epiphyseal trabecular width and bone area:tissue area in this study was statistically the same as treatment with alendronate and 80 mg/kg EPA, only alendronate with EPA at doses of 80 mg/kg and 320 mg/kg were able to increase the metaphyseal trabecular widths significantly in comparison to the MP group. It seems that alendronate combined with EPA treatment was able to produce a greater effect in increasing trabecular width when compared to treatment with alendronate as a single agent.

In the present study, MP did not significantly change the width of the tibial diaphysis in comparison to the control group, and there was no significant increase in the ratio of the marrow area:cortical area of the tibial diaphysis in the group treated with MP in comparison to the controls. Iwamoto *et al*., (2007) reported that treatment of rats with MP for four weeks induced cancellous osteopenia without affecting cortical bones significantly. However, the continuation of the same regimen for eight weeks resulted in cortical osteopenia in addition to cancellous osteopenia. This was manifested by a decrease in the percentage of cortical area and an increase in the percentage of marrow area in the tibial diaphysis. It appeared that cancellous osteopenia formed after a shorter amount of time or a smaller total amount of GC than cortical osteopenia.

Levels of CTX (a serum bone resorption marker), bone formation markers (osteocalcin and alkaline phosphatase), calcium, phosphorus (Pi) and creatinine concentrations were statistically similar between all of the groups in this study. In the study

performed by Wang *et al.*, (2002), the administration of MP at the doses of 2.5, 5, 10 or 20 mg/kg daily to growing rats for four weeks did not significantly change the values of serum calcium, phosphorus (Pi) and Pyridinoline (another resorption marker) in any group. In the same study, osteocalcin and alkaline phosphatase decreased significantly in only the groups treated with 10 and 20 mg/kg MP. In the present study, the calcium:creatinine and phosphorus:creatinine ratios were statistically similar between the different groups. In the study performed by Unoki *et al.*, (1995), the administration of 2.5 mg/kg prednisolone to rats six times a week for eight weeks did not significantly change the levels of urinary calcium and phosphorus (Pi) in comparison to the control group. It seems that in the present study, the histomorphometric parameters were more appropriate indicators of the detrimental changes to bone due the administration of GC to rats, at least in cancellous bone.

In conclusion, it seems that the administration of EPA in combination with alendronate can improve the beneficial effects of treatment with alendronate alone on the levels of bone loss associated with GC in rats.

Acknowledgments

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