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The influence of simvastatin in rats mandible and femur bone mass under Freund's adjuvant arthritis

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SUMMARY

Objectives. Complete Freund's Adjuvant (CFA)-induced arthritis in rats has been used widely as a model of rodent arthropathy and polyarthritis followed by osteoporosis, decreased bone formation and increased bone formation.

Osteoporosis is characterized by rapid reduce of bone mass affecting more than 100 million people worldwide. Periodontitis a chronic inflammatory, of multifactorian origin disease has been associated with general osteoporosis. Protective bone-specific anabolic and antiresorptive effects of HMG-CoA reductase inhibitors have also been evaluated in normal and osteoporotic bone.

Aim. The aim of the study was to investigate mandible and femur bone density in Freund's adjuvant induced arthritis rats under the influence of simvastatin.

Methods. Three groups (A, B, C) of 7 Wistar male rats each aged 3 months, (292±48.38 g) were used. A control. Group B and C subjected experimental arthritis via complete Freund's adjuvant injected in right paw. Group C was treated with simvastatin 0.5 mg/kg/daily po 14 days. Femur, mandible were isolated and sizes parameters, biochemical serum findings and BMD were estimated.

Results. CFA established by paw diameter, adrenals and spleen weight increase and thymus weight decrease, while biochemical serum findings were also affected. Reduced femur, mandible weight and general bone mass parameters BMD evaluated via DEXA occurred and restored under simvastatin treatment.

Conclusions. CFA induced mandible and femur injuries are repaired by ssimvatatin treatment that could be therapeutically useful

Key words: simvastatin, mandible, femur, freunds-adjuvant arthritis.

INTRODUCTION

Osteoporosis is the most common disease affecting more than 100 million people worldwide, characterized by rapid reduce of bone mass and microarchitectural deterioration of bone tissue. Osteopenia and increased risk of fractures are often clinically associated with long term corticosteroid treatment, deficiency of gonadal hormone secretion, malignancies (mammal or prostate carcinoma) and generally disorders of the patients endocrine status. The sustaining fractures leads to increased mortality rates (after hip or vertebral fractures). Moreover, osteoporosis has been associated with rheumatoid arthritis (RA) and periodontal disease, a chronic inflammatory, of multifactorian origin morbidity leading eventually to loss of the supporting structures of the teeth, including the jaw alveolar bone resorption. More severe manifestation specially of edentulous jaw bone resorption is observed in patients with diminished body mass index (1). Homeostatic bone remodelling requires the coordinated actions of the bone-resorbing osteoclast (OCL) and the bone-forming osteoblast (OB) (2).

Three major forms of bone loss have been described in RA. Focal articular bone erosions, a

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hallmark of RA, occur early in this disease and are associated over time with significant morbidity, periarticular bone loss, occurring adjacent to inflamed joints; and generalized osteoporosis, leading to an increase in fracture risk. Similar skeletal degenerative injuries occurs in destruction of the alveolar bone and soft tissue attachment to the tooth (3). Freund's adjuvant-induced arthritis in rats that has been used as a model of rodent polyarthritis, pathological features as chronic inflammation, involvement of peripheral joints, polysynovitis and further destruction of cartilage and bone. Decreased bone formation and increased resorption have been demonstrated during the development of polyarthritis and trabecular bone formation rate decreased during the first 2 weeks after injection of Freund's adjuvant and can been reversed by corticosteroids administration (4). Although there is an increased evidence that osteoporosis, and the underlying loss of bone mass characteristic of this disease, is associated with bone jaw resorption and tooth loss however the association both of them remains controversy (5).

Studies in human arthritis disease and in animal experimental models have as well, identified the osteoclast as the predominant cell type mediating bone loss. Many of the cytokines implicated in the inflammatory processes in rheumatic diseases have also been demonstrated to impact osteoclast differentiation and function probably directly, by acting on cells of the osteoclast-lineage (6, 7).

The integrity of the skeleton requires a dynamic balance between bone formation and bone resorption, which are controlled by hormones and cytokines. When bone resorption exceeds bone formation, diseases of bone metabolism such as postmenopausal osteoporosis can result (Riggs and Melton, 1992) (8). Communication between bone-forming osteoblasts and bone-resorbing osteoclasts is essential, and coupling of bone resorption to bone formation is necessary for the maintenance of healthy bone.

Moreover 3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase is the rate-limiting enzyme in the mevalonate pathway that provides essential intermediates for the biologic functions of growth-related proteins possessing anabolic and antiresorptive effect, as is suggested by a number of studies (9, 10). Protective bone-specific anabolic and antiresorptive effects of HMG-CoA reductase inhibitors have been also referred (11). HMG-CoA-reductase inhibitors are hypolipidaemic agents, with anabolic and antiresorptive effect and stimulate bone formation.

DEXA (Dual Energy X-ray Absortiometry) scans are primarily used to evaluate bone mineral density. DEXA uses X-rays to assess bone mineral density. However, the radiation dose is approximately 1/10th that of a standard chest X-ray. In some communities, a CT scan with special software can also be used to diagnose or monitor low bone mass (QCT).

This study focuses on the action of statins in the bone density of femur and mandible in Freund's adjuvant induced arthritis since statins inhibit the synthesis of mevalonate, stimulate the proliferation and differentiation of bone-forming osteoblasts, increasing bone formation *via* DEXA use and estimation of bone measurement parameters.

MATERIAL AND METHODS

21 Wistar male rats aged 3 months, weighting 292±48.38 g from the animal colony of the Athens Cancer Hospital St Savvas were randomized in three groups (A, B, C). The animals were housed under 12/12h light/darkness and they access water and food *ad libitum*. They are treated according the Guidelines of Care and Use of Laboratory animals (12).

Group A was the control while the experimental group B and C were injected with Complete Freund's adjuvant 0.1 mg/ml in the right-hind paw [Sigma-Aldrich 1 mg of *Mycobacterium tuberculosis* (H37Ra, ATCC 25177), heat killed and dried, 0.85 mL paraffin oil and 0.15 mL mannide monooleate]. Group C was treated with simvastatin (Zocor® – Merck) 0.5 mg/kg/daily *po via* a gastroesophageal catheter. The experimental duration was 14 days. The animals were sacrificed by decapitation, blood was collected from the jugular vein, centrifuged (3000 Reps/min) in order to obtain serum for further investigation (serum Albumin levels and rheumatoid factors). Thymus, spleen, adrenals, femur and mandible were isolated and weighted. Furthermore the volume and the specific weight of each bone were estimated. Femur, mandible was isolated and sizes parameters, BMD (Bone

Table 1. Rats body and organs parameters of Freund's adjuvant induced arthritis

Parameters	Groups X, mean±SD		
	A	B	C
Animals body weight on sacrifice day (14 d), g	310±9	208±9	277±12
Paw diameter, mm	0.62±0,14	1.74±0.51	1.52±0.18
Adrenals weight, mg	0.052±0,011	0.068±0.008	0.059±0.012
Spleen weight, mg	0.55±0.01	0.82±0.03	0.69±0.1
Thymus weight, mg	0.112±0.01	0.051±0.004	0.8 4±0.05

mass density) via dual energy X-Ray absorptiometry (DEXA) were estimated while biochemical serum findings were measured as well. Furthermore femur ash was obtained after drying by heating for 24 h at 600°C.

1 day before sacrifice rats were housed in metabolic cages and urine was collected in order to estimate urine 24h hydroxyproline levels using the Hypernosticon kit (Organon-Teknika, Boxtel, Holland) (13).

Tumor Necosis Factor (TNF-α) was measured by Rat TNF alpha ELISA Ready-SET-Go eBioscience, Ltd and IL-1β were estimated quantitatively by ELISA R&D Systems Europe, Ltd.

Albumin and total proteins were estimated byphotometric method via Advia Analyzer 1200 Siemens.

Statistical analysis was performed by A NOVA and t-test.

RESULTS

The animals of group B and C were affected by the complete Freund's adjuvant arthritis and demonstrated the relevant signs. The arthritis changes appear at the end of second week after adjuvant injection (B). Symptoms are present as redness, swelling, deformity of hind paw and are in absence in the control animals (A). The paw diameter was increased under CFA statistically significant ($p<0.001$), while simvastatin reduce it also with statistical significance ($p<0.05$). Moreover simvastatin treatment (C) ameliorate the arthritic status eg., the decreased thymus ($p<0.05$), enhancement of adrenals ($p<0.05$) and tend to restore spleen weight ($p<0.5$) (Table 1). The changes of the experimental arthritis were obvious by decrease of plasma albumin and total proteins levels ($p<0.01$), while enhancement of TNFa and IL-β ($p<0.001$) of group B animals (CFA) occur as is shown in Table 2. The diurnal urine hydroxyproline excretion was enhanced by CFA ($p<0.01$) and ameliorated after simvastain($p<0.05$) (Table 2).

The bone parameters were similarly affected under Freund's adjuvant arthritis. Femur specific weight ($p<0.05$) and the femur ash content ($p<0.01$). was decreased in B (CFA) group compared to the control and *via* simvastatin treatment tend to regain their initial values. The ratio femur ash/diurnal urine hydroxyproline is statistically reduced ($p<0.01$) in CFA group, ameliorated *via* simvastatin. Similarly mandible specific weight ($p<0.5$) and the mandible ash content ($p<0.5$). was decreased in B (CFA) group compared to the control and *via* simvastatin treatment tend to regain their initial values.

The DEXA measurements resulted a significant decrease of BMD in B group versus controls ($p<0.001$), while simvastatin's treatment decrease the severity of BMD lose. Statins treatment in general tend to restore both laboratory and clinical parameters (Table 3).

DISCUSSION

Although Freund's adjuvant arthritis as an experimental model does not completely mimic human rheumatoid arthritis, however it is a valuable model in the study of arthritis, because it shows some common characteristics of human rheumatoid arthritis, eg proliferative synovial tissue, subcutaneous nodules, and both cartilage and bone destruction (14-16).

The increase in paw volume of the injected foot represents a primary inflammatory phase which appeared from Day 3 to 10 after adjuvant injection. It seems that the presence of immune disorders may be responsible for the decrease of body weight in the experimental groups and statin treatment tend to prevent body weight loss. Total serum proteins and albumin are decreased during adjuvant arthritis in the same way as in conjunctive tissues diseases. Simvastatin seem to excert anti-inflammatory effect, since ameliorate both paw inflammation and proteins profile (17).

In rheumatoid arthritis are observed focal bone erosions and anatomical disorder of the joint component (pannus, cortical bone subchondral bone marrow space) (18). In addition it has been demonstrated that

Table 2. Laboratory findings of Freund's adjuvant induced arthritis in rats under simvastatin treatment

Parameters	Groups X, mean±SD			
	A	B	C	p
Total proteins, mg/dl	6.9±0.3	5.2±0.4	6.2±0.3	A/B p<0.01
Albunin, mg/dl	3.6±0.1	2.7±0.1	3.5±0.2	A/B p<0.01
TNF-α, pg/ml	520.9±3.5	847.5±18.9	697.4±20.2	A/B p<0.1, B/C p<0.05, A/C p<0.05
IL1-β, pg/ml	147.7±6.1	252.5±17.8	230.8±12.7	A/B p<0.01, B/C p<0.05, A/C p<0.05
Urinary hydroxyproline, mcg/24h	53.8±7	76.9±5	62.3±3	A/B p<0.01, B/C p<0.05, A/C p<0.05

lipopolysaccharide (LPS) increases the resorption of alveolar bone.

Periarticular bone loss occurs adjacent to inflamed joints and often precedes the appearance of focal bone erosions while osteopenia seems to occur in the majority of human articular inflammation cases (19-22). It is referred that the joint inflammation by arthritis induction in rats is followed by a delay in skeletal growth (23) and a substantial reduction in bone mass (24). Cytokines seem to be involved in these phenomena and may be responsible for the weight loss.

The evaluation of the bone mass parameters demonstrated that a decrease of femur BMD occurs in adjuvant animals (B) ($p<0.5$). This can be proved from the femur dimensions (bone weight, specific bone weight, DEXA values) and simvastatin treatment leads to regain the normal values (25) (group C).

Moreover the increased IL-β cytokine seems to play an important role at the level of inflammatory foci since the systemic administration of supraphysiological doses of interleukin-1β by Del Pozo et al 2005 is capable to induce moderate osteopenia in rats (26).

Inflammatory cytokines such as IL-1β and TNF-α are potent inducers of bone resorption. It is possible that these cytokines mediated both the increase paw volume and the decrease in BMD. The importance of TNF-α in humans can be elucidated by new data showing that treatment of rheumatoid arthritis patients with TNF-α antagonists, with and without immunosuppressant drugs, results in substantial reductions of clinical symptoms as well as retardation of radiographic progression of bone loss. However it is not sure if the bone loss reduction can be attributed to the effect of TNF-α on osteoclast formation, or if it is a consequence of the inflammation reduction (27).

Similar findings are referred from other investigators describing reductions in bone mass and strength of the lumbar body in a model of secondary osteoporosis (28, 29).

The immunomodulatory role of statins has been investigated the last years. Macrophages can be activated by cytokine like Type-I interferon produced by activated T-cells to secrete numerous factors like toxic oxygen metabolites, neutrophil chemotactic factors, arachidonic acid metabolites, growth factors, fibrogenic cytokines involved in tissue injury (30).

Statins have been shown to decrease the T-cell proliferation and reduce inflammatory cytokines production both tumor necrosis factor-(TNF a) and Interleukin-1 (31, 32).

In addition, it appears that statins can disrupt the oxidative stress/inflammation cycle by decreasing the release of inflammatory mediators and lipid peroxidation.

Since oxidative stress is recently recognized in inflammatory diseases like RA to perpetuate tissue damage, statins also can be helpful in RA patients by this mechanism. Chronic administration of statins can inhibit the inflammation process (33). Mundy et al (1999) (34) reported that statins, act not only as cholesterol lowering agents but, may stimulate *in vivo* bone formation in rodents and increase new bone volume in cell cultures. In fact, a large number of studies demonstrate a relationship between statin administration and fracture risk (35-41)

Adami et al (42) suggested a lower incidence of fractures among statin users explained by the higher bone mass in subjects with higher LDL-C levels and statin intake. On the other hand, other investigators, suggested that this class of drugs may exert positive action as is proved in a retrospective study of diabetic patients statins users comparing their BMD versus non-users (43, 44).

Table 3. Simvastatin changes of Bone-mass parameters in rats under Freunds-adjuvant arthritis

Parameters	Groups X, mean±SD		
	A	B	C
Femur spec. weight, mg/cm^3	1.62±0.18	1.46±0.12	1.50±0.175
Femur weight/body weight, mg/g	2.86±0.95	2.93±0.7	2.57±0.32
Femur ash, mg	7.5±0.1	5.8±0.1	6.1±0.2
Mandible (left) spec. weight, mg/cm^3	1.62±0.128	1.57±0.151	1.60±0.143
Mandible (left) weight / body weight, mg/g	2.55±0.41	2.57±0.51	2.47±0.09
Mandible (left) ash, mg	4.72±0.13	3.93±0.15	4.58±0.12
Mandible (left) ash, mg / Urinary hydroxyproline, mcg/24h	0.09±0.02	0.05±0.03	0.076±0.04
Femur ash, mg / Urinary hydroxyproline, mcg/24h	0.14±0.01	0.075±0.02	0.097±0.07
BMD Femur, gr/cm^2	0.190±0.014	0.169±0.010	0.181±0.006
BMD Mandible, gr/cm^2	0.213±0.010	0.205±0.013	0.220±0.008

Recently, is confirmed that statins stimulate the expression of bone anabolic factors, promote osteoblast differentiation and mineralization.

The ash amount showed a reduction in femur of arthritic animals in comparison to the controls representing the mineral content of the bones. Statins inhibit bone resorption by affecting the mevalonate pathway. As it was expected statin treatment prevented the loss of femur mineral values (group C) (45).

Urine hydroxyproline levels were increased in both experimental groups (B, C) with statistical difference from the control while group C tend to regain initial content Hydroxyproline excertion may serve as an index of the osseous tissue catabolism. The ratio femur ash / urine hydroxyproline was decreased in group B and C in comparison to controls ($p<0.05$) and group C tend to norm indicated a possible beneficial effect of statins on bone metabolism.

According the available technology, only a weak correlation between BMD/BMC in different sites of the skeleton and jawbones has been found, although there are studies indicating a correlation between bone mass in the mandible and skeleton in general (46, 47).

Previous studies have demonstrate a relationship between total bone mass reduction and alveolar bone loss in periodontal disease, as a significant correlation of alveolar bone resorption to the total bone mass reduction in patients with periodontal disease was found (48). A significant connection between periodontal connective tissue loss, as an indicator for periodontitis, and skeleton osteoporosis is confirmed by DEXA evaluation, especially in postmenopausal women (49).

Furthermore mandibular bone mass may serve as a considerable parameter for diagnosis, prevention and treatment of bone mass deficiency since under different kinds of experimental stress in mice a reduction of the mandible calcium content and the ratio calcium content per mandible volume was observed (50).

More over systemic factors as phenytoin and diazepam treatment may affect Ca^{++} content of the bones as mandible or femur inducing acceleration of vit D metabolism, increase of bone turnover rate, defective mineralisation, and disruption between osteoid formation and bone mineralization (51, 52).

In this study the femur changes could be followed by analogous in the mandibles dimensions alterations (without statistical significance).

The non injected bones regions as mandible, however, did not show significant bone loss

On the other hand the DEXA evaluation of the mandible of the present study show a decrease in the arthritis group that seems to be repaired after the statin's regiment.

Lindy et al. (53) demonstrated apart the effect of statins in skeleton they excert anti-inflammatory effect as well since it has been observed that subjects on simvastatin or on atorvastatin treatment showed 40% lower mean PIBI (Periodontal Inflammatory Burden Index) values than subjects without statin.

In vitro and in vivo studies suggested that statins may be useful for the treatment of a number of inflammatory conditions since they exhibit anti-inflammatory properties enhancing the expression of BMP-2 Bone morphogenetic protein-2 gene expression, reducing circulating C-reactive protein (CRP) levels and bone resorption through suppressing of matrix metalloproteinase 9 (MMP-9) expressions. More over stimulate the expression of bone anabolic factors, promote osteoblast differentiation and mineralization in MC3T3-E1 cells (Maeda et al, 2001, 2004) (54, 55). At last 3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase is the rate-limiting enzyme in the mevalonate pathway that provides essential intermediates for the biologic functions of growth-related proteins. According also to Fajardo (56) and Fentoğlu (57) investigations statins treatment may favored periodontal disease management

It may be concluded that the mandible and the femur affecting injuries by Freund;s adjuvant arthritis seems to be repaired by the simvastatins treatment encouraging its use in connective tissues diseases as a successful response.

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