



Original Article

Biometric, histomorphometric, and biochemical profile in atorvastatin calcium treatment of female rats with dexamethasone-induced osteoporosis[☆]



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ARTICLE INFO

Article history:

Received 6 June 2017

Accepted 27 June 2017

Available online 27 July 2018

Keywords:

Glucocorticoid

Diphosphonates

Alendronate

Alkaline phosphatase

Bone histomorphometry

ABSTRACT

Objective: To assess the effects of atorvastatin calcium in the treatment of dexamethasone-induced osteoporosis.

Methods: Osteoporosis induction consisted of the administration of an intramuscular dose of 7.5 mg/kg of body weight of dexamethasone, once a week for four weeks, except for the control animals (G1). The animals were divided into the following groups: G1 (control group without osteoporosis), G2 (control group with untreated osteoporosis), G3 (control group with osteoporosis treated with sodium alendronate 0.2 mg/kg) and G4 (group with osteoporosis treated with atorvastatin calcium 1.2 mg/kg). Serum alkaline phosphatase, bone alkaline phosphatase, and biometric and bone histomorphometric assessments were performed after 30 and 60 days of treatment onset.

Results: In relation to the biometric and histomorphometric analyses, at 60 days of treatment, G4 presented bone density (Seedor index), bone trabecular density, and cortical thickness of 0.222 ± 0.004 g/cm, $59.167 \pm 2.401\%$, and $387,501 \pm 8573$ μ m, respectively, with a positive and statistically significant difference ($p < 0.05$), in relation to G2. At 30 and 60 days of treatment, G4 presented statistically significant serum levels of alkaline phosphatase alkaline phosphatase ($p < 0.05$) that were higher than all groups (7.451 ± 0.173 μ g/L and 7.473 ± 0.529 μ g/L, respectively).

Conclusion: Treatment with atorvastatin calcium demonstrated the ability of this drug to increase osteoblastic activity and bone tissue repair activity, acting differently from alendronate sodium, which demonstrated predominantly antiresorptive activity.

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<https://doi.org/10.1016/j.rboe.2018.07.007>

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Perfil biométrico, histomorfométrico e bioquímico no tratamento com atorvastatina cálcica de ratas com osteoporose induzida com dexametasona

R E S U M O

Palavras-chave:
Glicocorticóide
Difosfonatos
Alendronato
Fosfatase alcalina
Histomorfometria óssea

Objetivo: Avaliar os efeitos da atorvastatina cálcica no tratamento da osteoporose induzida com dexametasona.

Métodos: A indução da osteoporose consistiu na administração de dexametasona na dose de 7,5 mg/kg de peso corporal, por via intramuscular, uma vez por semana durante quatro semanas, à exceção dos animais do grupo controle (G1). Os animais foram distribuídos nos seguintes grupos: G1 (grupo controle sem osteoporose), G2 (grupo controle com osteoporose sem tratamento), G3 (grupo controle com osteoporose tratado com alendronato de sódio 0,2 mg/kg) e G4 (grupo com osteoporose tratado com atorvastatina cálcica 1,2 mg/kg). Após 30 e 60 dias do início do tratamento dos animais, foram feitas as dosagens dos níveis séricos de fosfatase alcalina, fosfatase alcalina óssea, avaliação biométrica e histomorfométrica óssea.

Resultados: Em relação às análises biométricas e histomorfométricas, aos 60 dias de tratamento o G4 apresentou densidade óssea (índice Seedor), densidade trabecular óssea e espessura da cortical de $0,222 \pm 0,004$ g/cm, $59,167 \pm 2,401\%$ e $387,501 \pm 8,573$ μ m, respectivamente, com diferença positiva, estatisticamente significativa ($p < 0,05$), em relação ao grupo G2. Aos 30 e 60 dias de tratamento, o G4 apresentou níveis séricos de fosfatase alcalina óssea estatisticamente significativos ($p < 0,05$) e superiores a todos os grupos ($7,451 \pm 0,173$ μ g/L e $7,473 \pm 0,529$ μ g/L, respectivamente).

Conclusão: O tratamento com atorvastatina cálcica demonstrou a capacidade desse fármaco de aumentar a atividade osteoblástica e a atividade reparadora tecidual óssea, atuar de forma diferente do alendronato de sódio, que demonstrou atividade preponderantemente antirreabsortiva.

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Introduction

Glucocorticoid-induced osteoporosis is a serious and common complication in prolonged treatments with these drugs. A decrease in bone formation and an increase in its resorption are commonly observed in these cases.¹

This is a progressive disease that courses with the structural deterioration of bone tissue, leading to fragility and increased susceptibility to fractures caused by bone mass reduction and increased bone turnover.²

The treatment of glucocorticoid-induced osteoporosis aims at avoiding future fractures that decrease the quality of life. Bisphosphonates, among them alendronate sodium, act predominantly by inhibiting the resorption of bone tissue and are currently used in anti-osteoporosis drug therapies.^{2,3}

According to Drake et al.,⁴ alendronate sodium is approved for clinical use in the prevention and treatment of glucocorticoid-induced osteoporosis; its efficacy is higher when vitamin D and calcium levels are adequate.

Alendronate sodium has been reported to reduce bone loss in patients treated with moderate to high doses of prednisone for heterogeneous conditions.^{3,5}

However, alendronate sodium, despite reducing the incidence of fractures, does not lead to bone formation gains. Moreover, its prolonged use has shown several side effects, such as subtrochanteric femoral fractures, mandible

osteonecrosis, and esophageal irritation, among others, hindering patient adherence to treatment.^{4,6}

In a dyslipidemia model with ovariectomized rats, Lin et al.⁷ found that atorvastatin, clinically used in the treatment of dyslipidemias, not only decreased serum lipid levels but also promoted biomechanical bone improvement and increased collagen in the bone tissue.

Some recent studies have reported the role of statins in bone formation by stimulating the expression of bone morphogenetic protein (BMP)-2, which leads to osteoblastic differentiation and consequent bone formation. An increase in the transcription of the BMP-2 gene was observed, which is probably responsible for its effects.⁸

Based on these findings, it is believed that statins, if selectively directed to the bone, could have beneficial effects in the treatment of osteoporosis and fractures. These observations have aroused great interest in the scientific community; several studies have been conducted, demonstrating the role of statins in the improvement of bone density and in the reduction of the number of fractures.⁹

The present study is aimed at assessing, through biometric and bone histomorphometric evaluation, as well as biochemical markers, the effects of atorvastatin calcium in female rats with dexamethasone-induced osteoporosis.

Materials and methods

Experiment

This study used 48 adult Wistar female rats (*Rattus norvegicus*), with a mean weight of 200 g, sourced from the university's central vivarium; the animals received a commercially available feed and water *ad libitum* throughout the experiment.

All experimental procedures were approved by the Ethics Committee on Animal Use (Opinion No. 36/2016).

Throughout the experimental period, the animals remained in individual closed opaque polyethylene cages with a grid-shaped stainless steel lid. The animals were kept in an air-conditioned environment with a 12-h light cycle; the cages were sanitized every two days.

After one week of adaptation, osteoporosis induction was initiated with intramuscular glucocorticoid dexamethasone at a weekly dose of 7.5 mg/kg body weight for four weeks in animals of all groups, with the exception of the 12 animals from group 1 (G1 – control without osteoporosis).

The animals submitted to osteoporosis induction were then randomly distributed into three experimental groups of 12 animals each, as follows: G2 (control group with untreated osteoporosis), G3 (group with osteoporosis treated with alendronate sodium 0.2 mg/kg),¹⁰ and G4 (group with osteoporosis treated with atorvastatin calcium 1.2 mg/kg).¹⁰ The medications were administered daily by oral gavage, using appropriate cannulas.

Biochemical marker assessment

At 30 days and 60 days after treatment initiation, six animals from each group were anesthetized, and a 5 mL blood sample was collected (through abdominal laparotomy and subsequent vena cava puncture) to assess serum alkaline phosphatase (ALP) and alkaline phosphatase bone isoenzyme (ALP-BI).

For the laparotomy procedure, an intramuscular solution of 0.05 mL of ketamine hydrochloride (1 g/mL) with 0.05 mL of xylazine hydrochloride (23 mg/mL) was administered to each animal as a dissociative anesthetic medication.

ALP serum dosage was measured by spectrophotometry, using the Multiparametric Biochemistry Apparatus (Alizé), as well as a specific Bioclin[®] kit for use in automation.

For ALP-BI serum measurement, Beckman Coulter[®] Access Immunoassay System II chemiluminescence equipment was used, as well as a specific dosing kit from the same manufacturer.

Histomorphometric assessment

After the blood collection, at 30 days and 60 days after treatment initiation, six animals from each group were euthanized with anesthetic overdose (sodium thiopental 30 mg/kg).

The left femurs were harvested through dissection and placed in 10% neutral buffered formaldehyde for 72 hours for fixation; they were subsequently decalcified and processed for

histological study according to protocol and stained with Masson's trichrome staining.

To calculate the density of trabecular bone and bone cortical thickness, a bone sample from the subchondral region of each histological section was obtained using an Olympus BX 41[®] optical microscope (Tokyo, Japan) equipped with a digital camera (TCL-984 P[®]). The images were obtained using a 10 X objective lens. These images were analyzed in the open source image analysis software J (developed by Wayne Rasband of the Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, United States).¹¹

In the images obtained, the thickness of the cortical bone was measured in μm . The area composed of trabecular bone was calculated by the ratio between the area occupied by organic bone matrix and the total area of the image.

Biometric analysis

The length of each femur was measured with a Metrotools[®] 150 mm digital caliper and 0.01 mm/0.0005 resolution. Furthermore, each dissected femur was weighed on a A.Cientifica/EduTec[®] EEQ9003F-B digital analytical scale with a capacity for 220 g and reading accuracy of 0.1 mg. The Seedor index was calculated by dividing the weight of each femur by its respective length; the result was expressed in g/cm.¹²

Statistical analysis

The biological assay was performed according to a completely randomized design that consisted of four treatments and six replicates. The Shapiro-Wilk test was used to assess the normality of the variables ($p < 0.05$). The groups were compared among themselves using the Tukey test, with a 5% probability. The results were expressed as means $\pm$ standard deviations.

Results

The results of the biometrical evaluations (the ratio between weight and femur size [Seedor index; Fig. 1]) and histomorphometric measurements (trabecular bone density [Fig. 2] and thickness of the cortical bone [Fig. 3]) demonstrated that, during the entire experimental period, there was a progressive loss of bone structure that characterized the process of osteoporosis induction in the group that received the dose of 7.5 mg/kg dexamethasone and did not receive treatment (G2).

When compared with the group with untreated osteoporosis (G2), the animals in the group treated with alendronate sodium (G3) presented statistically significant differences regarding the Seedor index (Fig. 1), trabecular bone density (Fig. 2), and thickness of the cortical bone (Fig. 3), both at 30 and 60 days of treatment; the values in G3 were statistically similar to those of the control group (G1), demonstrating the ability to prevent osteoporosis induction with the action of dexamethasone in the fast phase, in which there is a predominance of increased osteoblastic activity.

Only at 60 days of treatment did the atorvastatin calcium group (G4) present the Seedor index (Fig. 1), trabecular bone

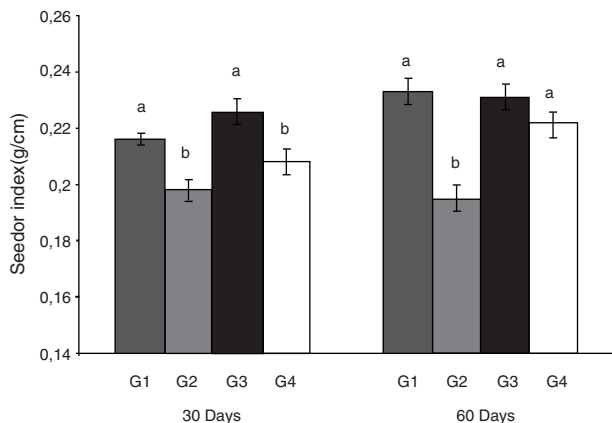


Fig. 1 – Biometric evaluation. Ratio between femoral weight and size (Seedor index) after 30 and 60 days of treatment. Results are expressed as mean $\pm$ standard deviation. In each period, mean values followed by different lowercase letters are differentiated by Tukey's test ($p < 0.05$). G1, control group without induction of osteoporosis; G2, control group with untreated osteoporosis; G3, control group with osteoporosis treated with alendronate sodium; and G4, group with osteoporosis treated with atorvastatin calcium.

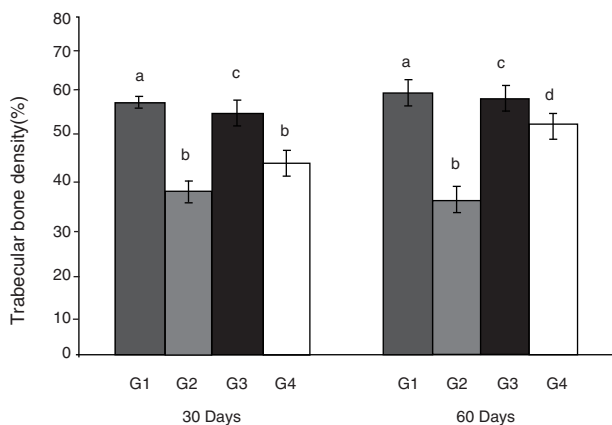


Fig. 2 – Histomorphometric assessment. Femoral trabecular density after 30 and 60 days of treatment. Results are expressed as mean $\pm$ standard deviation. In each period, mean values followed by different lowercase letters are differentiated by Tukey's test ($p < 0.05$). G1, control group without induction of osteoporosis; G2, control group with untreated osteoporosis; G3, control group with osteoporosis treated with alendronate sodium; and G4, group with osteoporosis treated with atorvastatin calcium.

density (Fig. 2), and bone cortical thickness (Fig. 3) statistically significant differences in relation to the group with untreated osteoporosis (G2), in addition to values closer to the control group (G1), demonstrating a better ability of this drug to act in the late phase of dexamethasone-induced osteoporosis.

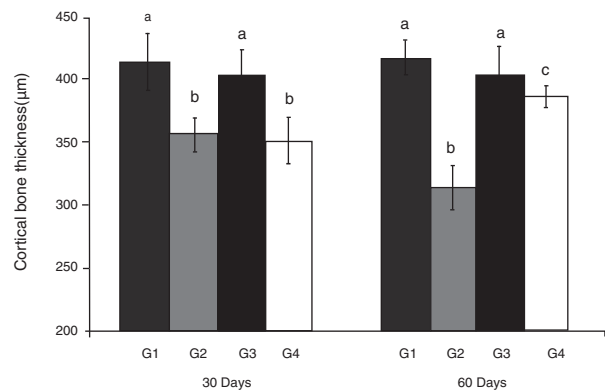


Fig. 3 – Histomorphometric assessment. Femoral cortical bone thickness after 30 and 60 days of treatment. Results are expressed as mean $\pm$ standard deviation. In each period, mean values followed by different lowercase letters are differentiated by Tukey's test ($p < 0.05$). G1, control group without induction of osteoporosis; G2, control group with untreated osteoporosis; G3, control group with osteoporosis treated with alendronate sodium; and G4, group with osteoporosis treated with atorvastatin calcium.

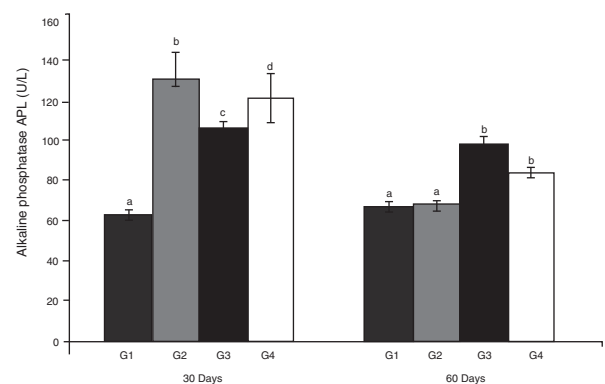


Fig. 4 – Serum levels of alkaline phosphatase (ALP) after 30 and 60 days of treatment. Results are expressed as mean $\pm$ standard deviation. In each period, mean values followed by different lowercase letters are differentiated by Tukey's test ($p < 0.05$). G1, control group without induction of osteoporosis; G2, control group with untreated osteoporosis; G3, control group with osteoporosis treated with alendronate sodium; and G4, group with osteoporosis treated with atorvastatin calcium.

After 30 days of treatment, serum ALP levels increased in all groups who underwent osteoporosis induction (G2, G3 and G4) when compared with the group without osteoporosis (G1; Fig. 4).

At 60 days of treatment, serum ALP levels were significantly decreased in all osteoporotic groups, although the groups treated with alendronate sodium (G3) and atorvastatin

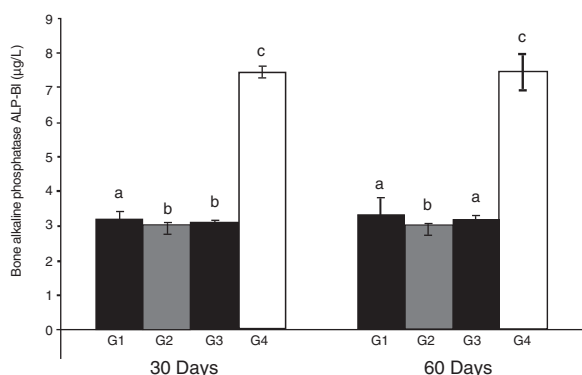


Fig. 5 – Serum levels of bone alkaline phosphatase (ALP-BI) after 30 and 60 days of treatment. Results are expressed as mean $\pm$ standard deviation. In each period, mean values followed by different lowercase letters are differentiated by Tukey's test ($p < 0.05$). G1, control group without induction of osteoporosis; G2, control group with untreated osteoporosis; G3, control group with osteoporosis treated with alendronate sodium; and G4, group with osteoporosis treated with atorvastatin calcium.

calcium (G4) remained above the parameter of normality (G1; Fig. 4).

At 30 and 60 days of treatment, a non-match between the serum ALP and ALP-BI levels was also observed (Figs. 4 and 5).

In the group with untreated osteoporosis (G2) a progressive reduction of serum ALP-BI was observed at 30 and 60 days of treatment (Fig. 5).

During the entire experimental period, the group receiving alendronate sodium (G3) presented serum ALP-BI levels below the normality parameter (G1), thus demonstrating the low potential for osteoblast activity induction of this drug (Fig. 5).

The atorvastatin calcium group presented significantly higher values of ALP-BI, both at 30 and 60 days of treatment; these values were even higher than those from the control group (G1; Fig. 5).

Discussion

According to Ferreira Junior et al.,¹⁰ histomorphometric bone evaluation is an extremely valuable method for dynamic assessment of the bone remodeling process, as it estimates precisely the extent of bone loss, calcification rates, and formation of bone tissue, while also being capable of identifying osteometabolic changes, such as osteoporosis.

Dexamethasone-induced osteoporosis occurs in two phases: a rapid phase, where bone mineral density (BMD) is reduced, presumably due to excessive bone resorption (osteoclasts), and a late, progressive phase, in which BMD decreases due to the impaired bone formation (osteoblasts and osteocytes).¹³

In the late phase, glucocorticoids decrease osteoblast count and their action. This leads to a suppression of bone formation, a central feature in the pathogenesis

of glucocorticoid-induced osteoporosis. Glucocorticoids decrease the reproduction of osteoblast lineage cells, reducing the number of cells that can differentiate into mature osteoblasts. Furthermore, glucocorticoids impair osteoblast differentiation, as well as their maturation.^{13,14}

According to Amaral et al.,² serum ALP levels include the levels of the bone-specific alkaline phosphatase isoform known as ALP-BI, which is secreted by osteoblasts and may present an increase in bone remodeling and therefore should follow this profile.

However, according to Allen,¹⁵ the interpretation of total ALP is complicated, as it can be affected by bone, intestinal, renal, and hepatic variables, hindering its use as a bone remodeling marker.

According to Amaral et al.,² the progressive reduction of serum ALP levels is caused by direct glucocorticoid action, causing a reduction in osteoblast count and activity, in addition to resulting in the reduction of bone matrix replacement, leading to the suppression of osteoprotegerin (OPG) production, which, at normal levels, limits the release of pro-osteoclastogenic enzymes.

The positive results in bone structure preservation observed in the group treated with alendronate sodium (G3), despite the low osteoblastic activity (indicated by ALP-BI serum levels), demonstrates a preponderance for resorptive inhibitory activity that contrasts with the rapid phase (increased bone resorption) of dexamethasone action.^{7,13,14}

The main mechanism of action of bisphosphonates is due to a very high affinity of the mineral component of bone tissue that binds hydroxyapatite crystals in a stable manner, inhibiting their breakdown and, thus, suppressing effectively bone resorption.¹⁶

In addition, nitrogen bisphosphonates (alendronate, risedronate, ibandronate, pamidronate, and zoledronic acid), apart from presenting an anti-resorption capacity through the connection with hydroxyapatite, act predominantly by inhibiting the activation of farnesyl diphosphate synthase in the mevalonate pathway, leading to osteoclast apoptosis, with a consequent decrease in bone resorption.⁷

According to Issa et al.,¹⁷ statins have been shown to reduce bone resorption and increase BMD and bone formation.

The ability of statins to act on bone resorption can be explained by a mechanism of action similar to that of nitrogenous bisphosphonates, which act on the mevalonate pathway and inhibit the action of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which results in the reduction of osteoclastic activity, thus modulating bone resorption.⁷

The increase in ALP-BI serum levels caused by atorvastatin calcium corresponds to the capacity of the statins to stimulate osteoblastic activity, demonstrating their greatest potential to act in the late phase of osteoporosis induction by dexamethasone.¹⁷

According to Alam,⁸ statins stimulate an increase in the transcription of the BMP-2 bone morphogenetic protein gene, with a consequent increase in BMP-2 expression, which leads to osteoblastic differentiation and a consequent increase in bone formation.

Lee et al.,¹⁸ in a study with lovastatin, also observed an increased bone alkaline phosphatase activity, bone matrix mineralization, and *in vitro* bone cell osteogenesis.

Furthermore, statins act on bone tissue by increasing the expression of vascular endothelial growth factor (VEGF). Several variants of VEGF, as well as their receptors, are expressed in osteoblasts, in which the presence of this cytokine has been shown to induce increased levels of bone alkaline phosphatase activity, as well as improve the response to parathyroid hormone (PTH).^{19,20}

Kaji et al.²¹ reported that Smad3, a key molecule in the transduction of TGF- β signaling to the nucleus, promotes an increase in the production of type I collagen (Col1), in ALP-BI activity, and in the mineralization ability of osteoblastic cells in rats. In addition, both PTH and glucocorticoids modulate the Smad3 pathway in osteoblastic cells, independently from TGF- β .^{19,21}

In osteoblastic cell culture, statins increased Smad3 levels independently of the induction of the TGF- β -receptor. Moreover, osteoblast apoptosis was suppressed through the TGF- β -Smad3 pathway.^{19,21}

Conclusion

Through biometric and histomorphometric analyzes, the induction of osteoporosis by dexamethasone was confirmed; it was also possible to assess the positive evolution of both the alendronate sodium group and the atorvastatin calcium group.

Treatment with atorvastatin calcium promoted significant positive changes in serum levels of ALP-BI and demonstrated the ability of this drug to increase osteoblastic activity and tissue repair activity, acting differently from alendronate sodium, which has been shown to act predominantly as an anti-resorptive agent.

Serum ALP levels that did not correspond to ALP-BI bone alkaline phosphatase may have been affected by intestinal, renal, and hepatic variants, hindering its use as a marker for bone tissue activity.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

To the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq]), the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior [Capes]), and the Graduate Program in Applied Biochemistry of UFV, for supporting this research.

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