



## Original Article

# Evaluation of simvastatin in the process of fracture healing in tibiae of rats

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## A B S T R A C T

**Objective:** Evaluate the effects of simvastatin in the process of fracture healing in rat tibia. **Methods:** Thirty-six rats were subjected to diaphyseal fracture of the leg bones and divided in the statin group (GE) and control group (GC), being subdivided into three subgroups according to days post-fracture (7<sup>th</sup>, 14<sup>th</sup> and 28<sup>th</sup> day) to assess bone healing. In GE was administered by gavage a solution of simvastatin to the sacrifice. In the control group was administered saline by the same route of SG. Immobilization was not used. After the sacrifice was made to limb amputation in the distal femur and conducted the clinical, radiological and histological analysis. Clinical evaluation was made as to the mobility of the fracture. Then the samples were radiographed and evaluated for callus diameter. Histological examination was performed with cuts of 5 micrometers and stained with hematoxylin-eosin, Masson's trichrome and Alcian blue pH 2.5. The level of significance to exclude the null hypothesis was 5%. **Results:** All GE animals showed greater stability of the fracture and higher callus area. There were no significant changes in the histological study. **Conclusion:** Simvastatin accelerates the consolidation process by increasing the callus, but does not alter the histology of the newly formed bone.

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## Introduction

Many studies have indicated that statins act towards remodeling and formation of bone tissue and are thus osteoinductive substances.

Mundy et al.<sup>1</sup> reported from studies on rodents that statins have anabolic effects on bone, with stimulation of bone tissue formation. Statins are believed to act by strengthening the expression of bone morphogenic protein-2 (BMP-2) in osteoblasts. However, in attempting to reproduce the experiments of Mundy et al.,<sup>1</sup> Maritz et al.<sup>2</sup> did not find the expected effect from statins. On the contrary, they reported that simvastatin produced an inhibitory effect with regard to bone formation, with increased bone reabsorption.

Several clinical investigations have reexamined the study series by means of observational and cohort experiments, in an attempt to evaluate the effect of statins on bone metabolism, fracture risk and bone consolidation.

Staal et al.<sup>3</sup> reported that statins act by inhibiting the mevalonate pathway. Mevalonate is the precursor for formation of steroids and the isoprenoids of steroids. These are involved in cell membrane biogenesis, DNA reproduction and protein glycosylation in a variety of cells. Statins inhibit the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA), thereby reducing cholesterol synthesis, which is required for conversion of HMG-CoA to mevalonate, and this is the same pathway as in bisphosphonate action. In acting to diminish mevalonate synthesis, statins interfere with cell proliferation and activity.

Baek et al.<sup>4</sup> studied the effect of simvastatin on the proliferation and differentiation of cells of the bone marrow stroma in culture medium. They isolated mononuclear cells and cultured an osteoblastic lineage from the bone marrow of healthy volunteers. In the first culture medium, simvastatin diminished the average size of the colonies that give shape to fibroblastic units (colony-forming units, CFU-Fs) and emphasized calcification of the matrix. These cells were subcultured and the alkaline phosphatase activity of each group was measured in relation to time. Simvastatin increased the alkaline phosphates activity in a dose-dependent manner, and the effect was most evident over the initial periods of culturing. It was concluded from that study that simvastatin has a stimulatory effect on bone formation by means of osteoblastic differentiation.

Researchers from the Heart Protection study did not reported any difference in hospital treatment for the same type of fractures caused by car accidents, between the group that used simvastatin (40 mg/day) and the placebo group.<sup>5</sup>

Through these studies, contradictory results regarding the effect of statins on bone metabolism have been generated.<sup>6</sup> Moreover, the action of this drug on fractures, mineral density and bone remodeling in humans is uncertain. Thus, a path towards new studies has been opened up, through the need for further observational, clinical, randomized and experimental studies on statin use at appropriate doses.

The aim of this paper was to evaluate the effect of statins on fracture healing in rats.

## Methodology

Thirty-six adult male Wistar rats (*Rattus norvegicus albinus*; Rodentia: Mammalia), aged 45 to 65 days and weighing 200 to 300 g, were used. They were housed in the Laboratory of the Maranhão Academic League of Experimental Surgery (LabLacema), in polypropylene boxes (0.15 m<sup>2</sup>), with a maximum of five animals in each box. This study followed the standards of the Nomina Anatomica Veterinaria<sup>7</sup> and the ethical principles for animal experimentation (Cobea), and it had been approved by the Ethics Committee of the Veterinary Department of Universidade Estadual do Maranhão.

Each animal was anesthetized with ketamine at a dose of 75 mg/kg and xylazine at a dose of 8 mg/kg intramuscularly in the left gluteal region. Antisepsis was performed on the right hind leg using a degerming solution and iodine-based antisepsis.

To produce the tibial fracture, pliers with three pressure points were used, in accordance with the technique established by Otto et al.<sup>8</sup> The rats then remained housed in their respective boxes, without any type of immobilization or restriction of weight-bearing on the fractured limb.

The animals were distributed randomly by means of a draw into two groups of 18, which were named the statin group (SG) and control group (CG). Each group was divided into three subgroups (SSG and SCG) that were defined with numerical suffixes corresponding to the number of days between the surgical procedure and sacrifice, thus:

- SSG-07 and SCG-07: (sacrifice on the 7th day) – five rats in each subgroup;
- SSG-14 and SCG-14: (sacrifice on the 14th day) – five rats in each subgroup;
- SSG-28 and SCG-28: (sacrifice on the 28th day) – eight rats in each subgroup.

These intervals between fracture production and sacrifice of the animals followed the experimental model proposed by Padula et al.<sup>9</sup>

Each rat was weighed and identified in its box with stripes made using a CD pen on the animal's tail. The rat identified as R1 was marked with one transverse stripe, R2 with two transverse stripes, R3 with three transverse stripes, R4 with one longitudinal stripe and R5 was not marked but was only subjected to the stress using a pen without ink. For SSG-28 and SCG-28, there were four animals in four boxes (two in each subgroup), thus making a total of 16 animals, which were identified as R1 to R4.

The SG rats were subjected to oral gavage with a simvastatin solution at a dose of 20 mg/kg, 12 hours after fracture production, and this was repeated every 24 hours. In the CG, gavage was performed using placebo. Gavage was performed every day until the date of sacrifice.

The animals were sacrificed after being weighed, and this was done by administering a lethal intraperitoneal dose of barbiturate after induction of anesthesia using halothane, and the act was completed with a lethal dose of potassium chloride. The hind leg was amputated at the level of the middle

third of the femur in order to study the bone callus. The skin around the musculature of the tibia was then removed without damaging the muscle group.

The mobility of the fracture focus was clinically evaluated. Grade 0 was attributed to consolidated fractures, grade 1 to intermediate patterns of movement and grade 2 to cases of clear movement.<sup>10</sup> All the examiners were defined by means of a draw and they were unaware of which group they were going to examine. After the clinical evaluation of mobility of the fracture focus, the surgical specimen was fixed in 10% formol.

The area of the bone callus was assessed by means of analysis on digital radiographs, using the Auto CAD® 2007 software (Fig. 1). The entire callus area was delimited and measured in mm<sup>2</sup>.

For the histological analysis, the fractured tibiae and their surrounding soft tissues were decalcified for 12 to 15 hours and then dehydrated, clarified and embedded in paraffin. Sections of thickness 5 micrometers were cut sagittally to the fracture plane, using a microtome. These sections were stained with hematoxylin-eosin (HE), Masson's trichrome (MT) and Alcian blue pH 2.5 (AB). HE and MT were used to study the cellularity of the extracellular matrix, while AB showed the sulfated and non-sulfated acid proteoglycans, which reflect the chondrogenic activity. All the laminas were analyzed by means of optical microscopy. The stage of bone consolidation was determined in accordance with the classification system proposed by Allen et al.<sup>11</sup> (Chart 1).



**Fig. 1 - Use of Autocad® for calculating the area of the callus.**

Descriptive statistical techniques were applied, thus enabling exploratory analysis through parameter estimates (mean standard deviation and median) for all the variables investigated in this study. Following this, bone consolidation was assessed by means of the dependent variable (bone area) in relation to the independent variables of days and groups.

Considering that the sample size was small and that the data did not present normal distribution, the nonparametric Mann-Whitney test was performed. To analyze the histological data, quantitative assessment was performed using Fisher's exact test. For all the tests, the significance level for rejecting the nullity hypothesis was 5%.

**Results**

All the animals accepted the diet well and could move around after receiving the fracture. They presented claudication of the fractured limb after an average of six to eight days, and there was no difference between the statin group (SG) and the control group (CG). None of the animals presented an operative wound at the fracture site.

**Clinical analysis**

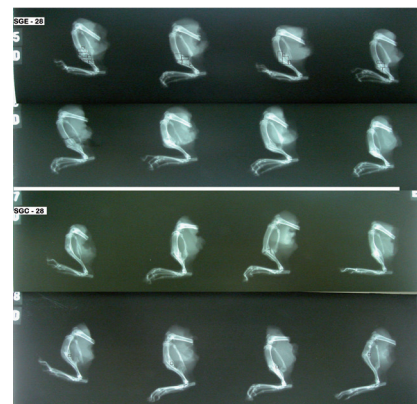
Regarding the mobility of the fracture focus after seven days, in SCG-07 there were four animals with intermediate mobility (grade 1) and one with clear mobility (grade 2), while in SSG-07 all five animals had intermediate mobility (grade 1).

In the subgroup evaluated 14 days after fracturing, there were four animals in SCG-14 without mobility (grade 0) and one with intermediate mobility (grade 1), while all five animals in SSG-14 had mobility grade 0.

All the animals that were evaluated 28 days after receiving the fracture presented mobility grade 0 on examination.

**Chart 1 - Histomorphological classification proposed by Allen et al. for staging bone consolidation.**

- Grade 4 Complete bone union
- Grade 3 Bone union incomplete due to presence of small quantity of cartilage in the callus
- Grade 2 Well-formed hyaline cartilage bridge can be seen, uniting the main fragments (complete cartilaginous union)
- Grade 1 Incomplete cartilaginous union, with retention of fibrous elements in the chondral plate
- Grade 0 Absent or delayed fracture repair, characterized by presence of cartilage between the fragments and remains of hematoma or other fluid (pseudarthrosis)



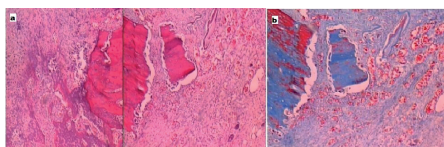
**Fig. 2 - Radiographs on the SSG and SCG subgroups on the 28<sup>th</sup> day.**

### Radiological analysis

All the animals in the subgroups that were sacrificed on the seventh day presented fractures of the tibia and fibula, thus demonstrating that the fracture closure took place in a standardized manner. No radiological bone callus was found in any of the rats at this time.

Among the subgroups that were sacrificed on the 14<sup>th</sup> and 28<sup>th</sup> days, bone calluses and bone bridges were seen in all the rats. In SSG-14, the mean area of the callus was 66.65 mm<sup>2</sup>, while in SCG it was 55.29 mm<sup>2</sup>. SSG-28 also had a greater mean area of bone callus than SCG-28, with values of respectively 67.46 mm<sup>2</sup> and 56.09 mm<sup>2</sup> (Fig. 2). Therefore, the simvastatin group presented a mean bone callus size that was greater than in the control group, both in the 14 and 28-day evaluations and in the overall assessment.

There was a significant difference in relation to callus area ( $p = 0.043$ ), such that the average area in the statin group (67.1 mm<sup>2</sup>) was greater than the area in the control group (55.4 mm<sup>2</sup>).



**Fig. 3 - Photomicrographs of sections from animals in the 14-day subgroups, grade 1, SSG-14. (a) Presence of many neofomed vessels in fibrous connective tissue, with presence of formation of immature bone tissue. H&E x4. (b) With MT.**

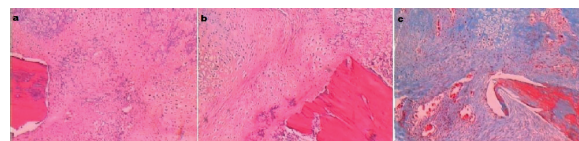
### Histological analysis

All the rats were histologically evaluated by the pathologist responsible, in a random manner without access to or knowledge of the control group, the group divisions or the respective days of sacrifice of each subgroup.

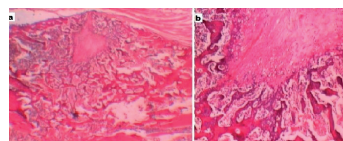
In SCG-07, there were elements of fibrous connective tissue, absence of or only small focal areas of bone tissue, with bone calluses of grade 1 observed in three animals and grade 0 in two. In SSG-07, there was fibrous connective tissue and cartilaginous tissue, with the periphery of the callus composed of elements of mature and immature bone tissue. In four animals, grade 1 bone callus was seen and in one, grade 0.

In SCG-14, four rats presented fibrous connective tissue and all of them had elements of chondrogenesis. There was grade 1 bone callus in four of these animals and grade 2 in only one. In SSG-14, three rats were classified as grade 2 and two as grade 1, and bone tissue was present on the periphery of the callus (Figs. 3 and 4).

In SCG-28, there was greater variation regarding the Allen classification. In this subgroup, one had bone callus of grade 4,



**Fig. 4 - Photomicrographs of sections from animals in the 14-day subgroups, grade 2. (A) SCG-14. Bone extremity with mature cartilaginous tissue, with complete union. HE x4. (B) SSG-14. Extremity with mature cartilaginous, forming complete hyaline bridge. HE x4. (C) SSG-14. Opposite extremity with vascular neofomation. MT x4.**



**Fig. 5 - Photomicrographs of sections from animals in SSG-28, grade 3. Formation of incomplete bone tissue due to presence of cartilaginous tissue in focal area (a - magnifying glass) and (b - x4).**

four grade 3, two grade 2 and one grade 1. In SSG-28, there was bone callus grade 3 in six animals (Fig. 5) and grade 1 in two. In all the animals of this subgroup, bone tissue with presence of osteocytes and osteoblasts was found.

In the quantitative evaluation, Fisher's exact test was used and no statistically significant difference was found between the groups.

### Discussion

The bone consolidation process is influenced by a variety of factors that have been studied in depth from the times of Hippocrates to the present day.

In the present study, simvastatin was chosen from among various inhibitors of HMG-CoA reductase because like atorvastatin and cerivastatin, it presents greater in vitro effects on bone than seen with lovastatin and pravastatin.<sup>3</sup>

Oxlund and Andreassen<sup>12</sup> found that treatment with simvastatin at a dose of 20 mg/kg orally partially impeded bone loss in female Wistar rats that were subjected to ovariectomy, and also increased cortical bone formation. Based on these authors' results, we used this dose taking into account simvastatin metabolism in the liver, in order to investigate its effect on bone tissue.

Some authors, like Skoglund et al.,<sup>13</sup> used much larger doses of the drug, of 120 mg/kg, in order to demonstrate the effects of statins on bone consolidation in animals. They concluded that animals only presented increased bone callus area and noted that simvastatin acts on the mechanism for mobilization of cells involved in the fracture repair process, thereby leading to increased bone callus size.

In our study, we also observed that the bone callus area increased, thus corroborating these authors' affirmation.<sup>13</sup> The acceleration of the consolidation process in the statin group may have occurred in SSG-07, but this could not be seen in our study because the callus is radiotransparent at this stage of radiographic evaluation. On the other hand, both SSG-14 and SSG-28 presented large areas of bone callus, thus demonstrating acceleration of the consolidation process.

However, in the study by Skoglund et al.,<sup>13</sup> the results relating to the callus in the rats treated with simvastatin for 14 days were better than ours, with a cross-sectional area 53% larger than that of the control. However, after 21 days of treatment, the fracture consolidation did not present any differences between the control group and simvastatin group. In our study, SSG-28 presented bone callus size greater than that of the control, thus disagreeing with the above authors.

The findings of Maritz et al.,<sup>2</sup> in which statins increased the bone metabolism rate, thus increasing both bone formation and bone reabsorption, seem to favor bone consolidation when statins are used.

We also agree with the findings of Tsubone et al.,<sup>14</sup> regarding the capacity of statins to promote bone formation and improve the consolidation process. These authors studied cerivastatin used in bone transplantations in rat tibiae and found through histological analyses that it increased the formation of new bone tissue and increase bone union.

As already described, the mechanism for inducing bone consolidation triggered by simvastatin is mediated by increased production of BMP-2. Therefore, it is possible to compare the results from studies that used BMP-2 rh in the consolidation process and those that used statins.<sup>15</sup>

Stein et al.<sup>15</sup> studied the effects of rhBMP-2 on consolidation of unstable and stable fractures of the tibia in rabbits, administered in biodegradable particles, in collagen gel or by injection. When rhBMP-2 was injected, the callus of mechanically unstable fractures developed more rapidly. In the same way as in our study on statins, these authors concluded that although BMP-2 accelerates the callus development rate and cortical bone union, it does not affect the quantity of bone and cartilage produced. In our study, there was no statistically significant difference regarding histomorphological classification when 7, 14 and 28 were compared. However, there were certain variations in histological grade between the different subgroups.

## Conclusion

Simvastatin promoted greater clinical stability for the fracture focus and increased the size of the bone callus, 14 and 28 days after the fracturing event, such that this difference was statistically significant. It also produced a difference in the histological analysis on the callus, but without statistical significance.

## Conflicts of interest

The authors declare that there was no conflict of interests in conducting this study.

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