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Tibolone, alendronate, and simvastatin enhance implant osseointegration in a preclinical in vivo model

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Abstract

Objectives: The objective of the study was to evaluate and compare the effect of different drugs such as simvastatin, alendronate, and tibolone for titanium implant osseointegration enhancement.

Materials and methods: Eighty female albino Wistar rats were equally divided into five groups: Group I (ovariectomy), Group II (sham ovariectomy), Group III (alendronate + ovariectomy), Group IV (simvastatin + ovariectomy), and Group V (tibolone + ovariectomy). Three months after ovariectomy, we performed bilateral titanium intramedullary nailing in all groups, followed by oral administration of alendronate, simvastatin, or tibolone for 12 weeks. Examinations included micro-CT, mechanical pull-out test, histology, and bone serum markers.

Results: Peri-implant micro-CT analysis showed a significantly higher overall bone tissue in tibolone compared to the ovariectomy group, while no significant difference was found between the treatment groups. Sham ovariectomy, alendronate, and tibolone groups had a higher body mass density compared to ovariectomy and simvastatin groups. All treatment groups had a greater thickness of the peri-implant compact bone layer compared to ovariectomy group, but the results were not statistically significant. Tibolone presented the highest values in pull-out test, but alendronate showed more consistently positive results compared to other groups. Osteocalcin had in the tibolone group almost three times the value in the ovariectomy group, but the results were not statistically significant.

Conclusion: The hypothesis that alendronate, simvastatin, and tibolone enhance the osseointegration process of intramedullary titanium implants in ovariectomized rats has been accepted, while tibolone could offer the best results.

KEYWORDS

animal experiments, bone regeneration, bone–implant interactions, drug delivery, guided tissue regeneration, pharmacology

1 | INTRODUCTION

Endosseous dental implants are frequently performed procedures worldwide for the treatment of partial or total edentation, with more than 5,000,000 being placed each year only in the United States (DiMatteo & Latanyshyn, 2014; Dym, 2015). This type of surgery improves the quality of life in most patients and has good implant survivorship represented by almost 91% at 10-year follow-up (de Moraes et al., 2015). Dental implants which are made up of titanium alloys require a good bone metabolism to allow for a proper bone ingrowth at the level of the implant, a process called osseointegration (Apostu et al., 2017; Buzatu et al., 2019; Carmen et al., 2014; DiMatteo & Latanyshyn, 2014; Osman & Swain, 2015). A good balance between the catabolic processes and anabolic processes is essential for a proper osseointegration process; still more research is needed to further understand the osseointegration process (Apostu, Lucaciu, Berce, Lucaciu, & Cosma, 2018; Yang et al., 2014).

An impaired osseointegration process can lead to fibrous healing at the recipient bone and implant interface, which clinically results in screw loosening (Hanif, Qureshi, Sheikh, & Rashid, 2017). This complication implies implant removal and replacement of the implant, where functional impairment, patient satisfaction, and functional outcome compared to primary interventions are worse (Apostu et al., 2017, 2018; Levin, 2008).

Multiple methods have been studied to improve the osseointegration process such as increasing the implant's biocompatibility and changes within the surgical technique, but the outcomes are limited (Apostu et al., 2018). Systemic drugs have been shown to enhance the bone metabolism process and therefore improve osseointegration of titanium implants (Apostu et al., 2017).

Two of the most commonly used systemic drugs shown to improve implant fixation in previous studies are alendronate and simvastatin (Apostu et al., 2017). Alendronate is a bisphosphonate that inhibits the mevalonate pathway, which prevents the formation of proteins necessary for osteoclast differentiation and function, while simvastatin is a lipid-lowering agent which also possesses a dual anti-anabolic and catabolic action on bone metabolism (Apostu et al., 2017). Both have proved to enhance osseointegration of titanium implants in an animal model; however, none of these previously described agents have been compared in the same study to decide which has the best result on osseointegration process and which is the best candidate for future clinical trials (Apostu et al., 2017).

Selective tissue estrogenic activity regulators (STEAR) represented by tibolone are currently used for the treatment of menopausal symptoms and prevention of postmenopausal osteoporosis. Although tibolone targets estrogen, progesterone, and androgen receptors, its effect on bone is mainly due to the activation of estrogenic receptors (Gambacciani & Levancini, 2014; Kloosterboer, 2004). We found no research to study the impact of tibolone on the osseointegration process of the titanium implants, but we consider that by suppressing the bone resorption process, tibolone could improve this process.

Due to a higher adherence rate of patients due to oral administration, low cost, low risk of adverse reactions, and the favorable effect found in animal studies, we consider that systemic drugs can play an important role in future prevention of one of the most common complications in dental implants, that is, fibrous encapsulation.

The study hypothesizes that the systemic administration of alendronate, simvastatin, and tibolone enhances the osseointegration process of titanium implants demonstrated by histological, micro-CT, and pull-out tests examinations.

2 | MATERIALS AND METHODS

2.1 | Animal model

The study received the Ethics Commission approval (no. 467/14.12.2016) as well as the Sanitary Veterinary and Food Safety Agency approval (no. 33/06.02.2017). We performed the experiments at the Centre of Experimental Medicine Cluj-Napoca, according to institutional, national, and European guidelines (Directive 2010/63/EU). A total of 80 female albino Wistar rats of 9–11 weeks old and weighing 200 ± 50 mg, born and raised at the same animal facility, without any genetic modification, were included in the study. The animals were kept at a temperature of 21°C and 12-/12-hr dark/light cycle. We provided standard pellet-food and water ad libitum. The rats were randomized into five groups: Group I (control + ovariectomy), Group II (control + sham ovariectomy), Group III (alendronate + ovariectomy), Group IV (simvastatin + ovariectomy), and Group V (tibolone + ovariectomy).

2.2 | Ovariectomy (OVX) and sham ovariectomy procedures

These procedures were performed 1 week following the allocation within groups and after the subjects were declared clinically healthy by a veterinary doctor (Figure 1). General anesthesia was performed using a cocktail of 80–100 mg/kg of ketamine and 10–12.5 mg/kg of xylazine intraperitoneally. After skin preparation and sterile draping, we made an abdominal midline incision distal to the xiphoid process. The dissection continued until both ovaries were exposed (Figure 2a) and excised after identification in the case of groups I, III, IV, and V (Figure 2b). Electrocautery was used to prevent abdominal bleeding (Figure 2b). The abdominal wall and skin were then sutured, followed by the topical application of an antibiotic (tetracycline). The sham ovariectomy procedure, performed in group II, consisted of the same surgical steps as the ovariectomy procedure, but we did not excise the ovaries after identification. Postoperatively, we administered analgesia in the provided drinking water.

2.3 | Intramedullary nailing

Three months following ovariectomy and sham ovariectomy procedures, we performed bilateral femoral intramedullary nailing in all of the groups (Figure 1). Following general anesthesia (as previously

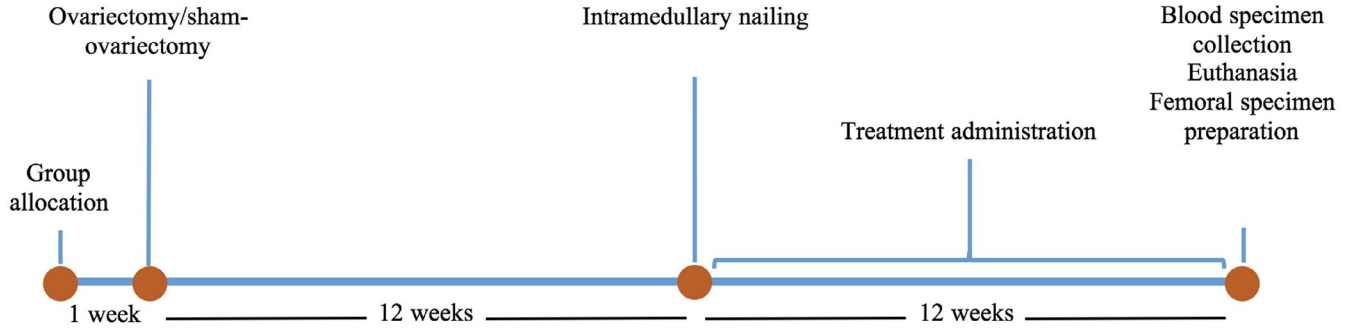
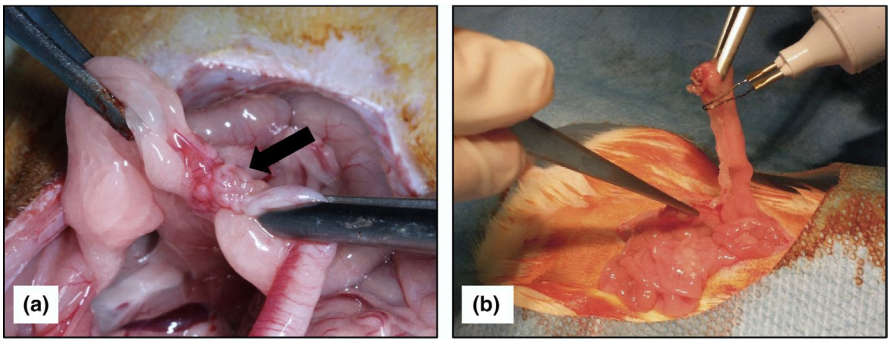


FIGURE 1 Timing of the project's main stages: group allocation, ovariectomy/sham ovariectomy procedures, intramedullary nailing, treatment administration, blood specimen collection, euthanasia, and femoral specimen preparation

FIGURE 2 Surgical intervention of ovariectomy: (a) exposure of ovaries (arrow); (b) excision of ovaries using electrocautery



described), the rats were weighed, and we made a longitudinal incision between the patella and the tibial tuberosity in full flexion of the knee. The patellar tendon was transversally incised, and the intercondylar notch was exposed (Figure 3a). The femoral canal was opened using sterile 18-gauge needles (Figure 3b). Sterilized intramedullary annealed $Ti_{90}Al_6V_4$ alloy nails (Goodfellow Cambridge Ltd.) of 1 mm ± 10% in diameter and 20 mm ± 10% in length, with an average roughness of 2.6 μm, were then inserted into the femur using the press-fit technique (Figure 3c). The patellar tendon and skin were sutured, followed by the topical application of an antibiotic (tetracycline). Postoperatively, we administered analgesia in the provided drinking water.

2.4 | Treatment

Groups I and II did not receive any drug, as they were only provided food and water ad libitum. In the case of treatment groups, oral administration began on the first postoperative day and continued for 12 weeks (Figure 1). We mixed one piece of food pellet with the required quantity of the dedicated drug, and no extra food was provided for 3 hr before administration. During the administration period, we placed the rats in individual cages. Group III was administered alendronate (Alendronate Sandoz®) at a dose of 3 mg/kg twice a week, Group IV received simvastatin (Simvastatin Terapia®) at a dose of 5 mg/kg daily, and Group V was administered tibolone (Livial®) at a dose of 1 mg/day (Carvalho et al., 2012; Chen, Li, Yang, Xu, & Xie, 2013; Du, Chen, Yan, & Xiao, 2009). For proper dosing of drugs, we weighted the rats daily, and the drug quantity was adjusted accordingly. Successful drug administration was considered if the subject completely consumed the food pellet mixed with the tested drug, and the result was noted daily in each subject.

2.5 | Collection of samples and euthanasia

Three months following intramedullary nail implantation, general anesthesia was performed as previously described, followed by the weighing of the rats and the collection of the blood samples by cardiac puncture (Figure 1). The animals were then euthanized by cervical dislocation (Figure 1). We noted the atrophy of the uterus and presence of ovaries. Afterward, the bilateral femurs were collected and placed in 10% formaldehyde until analysis (Figure 1). Right femurs underwent a micro-CT and mechanical pull-out test, while left femurs underwent histological examinations.

2.6 | Micro-CT examination

A Burker Skyscan 1172® device was used for micro-CT scanning at a resolution of 2000 × 2000 pixels. The region of interest (ROI) was a round shape with a diameter of 120 mm centered on the implant, and the length on the ROI consisted in 700 slices (9.48 mm of height) starting proximally from the distal metaphysis, using a standard threshold in each examination (Figure 4a-c). The following quantitative parameters were assessed using the specialized Bruker CTAn® software: bone volume (BV), percent bone volume (BV%), bone surface (BS), tissue surface (TS), bone surface/volume ratio (BS/VR), mean total

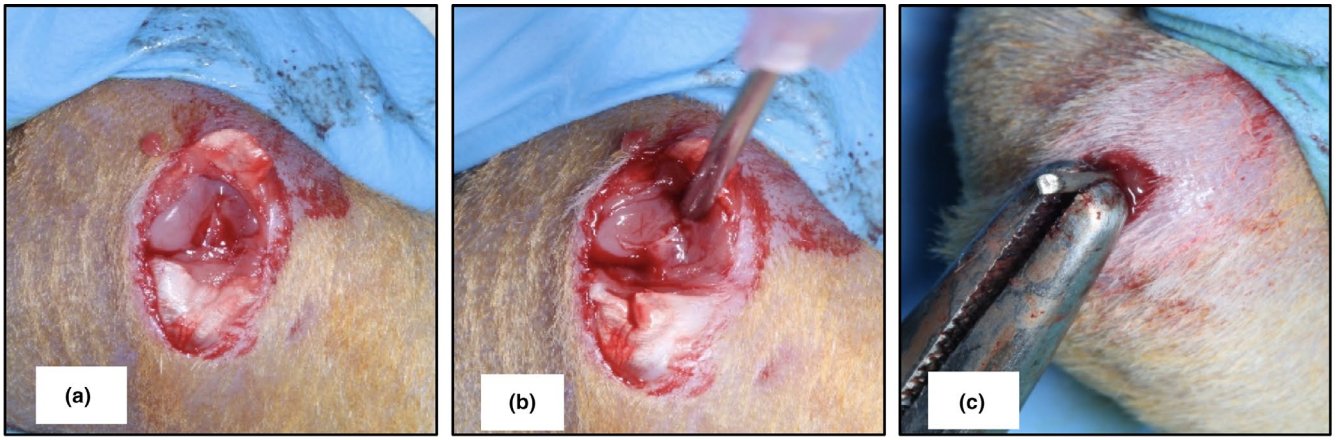


FIGURE 3 Surgical technique of femoral implantation of titanium implants: (a) exposure of intercondylar notch; (b) opening of the femoral canal; (c) insertion of the titanium implant

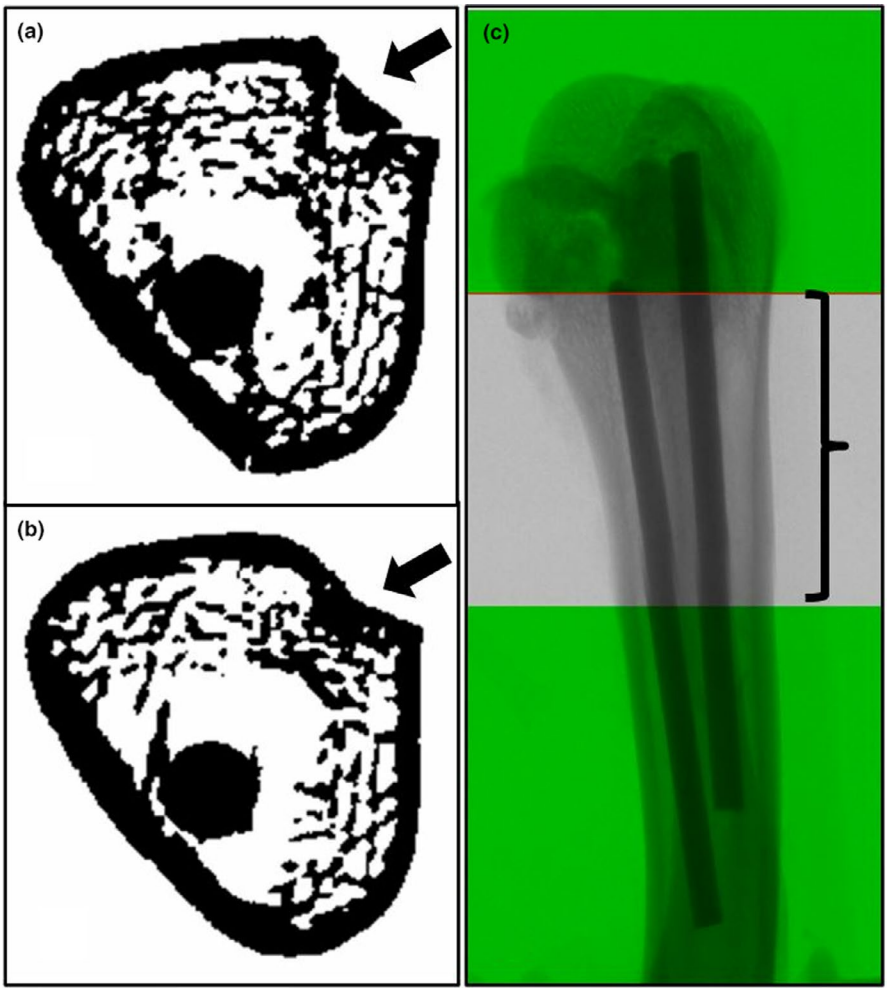


FIGURE 4 Determination of region of interest length for micro-CT analysis: (a) identification of femoral intercondylar notch (arrow); (b) distal starting point for region of interest is represented by the first sequence proximal to the intercondylar notch where the anterior cortical bone is continuous (arrow); (c) the region of interest analyzed is 700 slices proximal to the starting point (bracket)

cross-sectional bone area, cross-sectional thickness, trabecular diameter (TD), and trabecular number (TN). Bone mineral density (BMD) was also calculated after calibration using a dedicated phantom provided by Bruker®, and we examined the entire femur. The peri-implant bone formation was calculated using a formula described by Choi (Choi, Choi, & Yeo, 2018). The examinator performing the measurements was not aware of the allocation within groups.

2.7 | Histological analysis

The femurs were decalcified, and longitudinal sections along the implant were done. After exposure, the implants were then carefully extracted under a microscope to avoid damaging the surrounding bone. We then performed hematoxylin–eosin and Tricrom Masson stainings. Both stainings were analyzed at the 20× objective (200×

magnification) using a Leica DM750® microscope. We used ImageJ® software for the morphometric measurements. The thicknesses of the medial and lateral cortical bone tissue surrounding the implant were measured at 10 mm from the implant's distal extremity, and the average was calculated. Three separate measurements were done by different examinators who did not know the allocation within groups, and we noted the average.

2.8 | Mechanical pull-out test

The mechanical extraction test, also called the pull-out test, was done using a Zwick/Roell Z005® tensile testing machine. The femoral specimens were cut distally until 5 mm of the titanium implant was revealed. We then tightened the exposed implant in a pneumatic grip, and the maximal extraction force applied in the longitudinal axis needed to release the implant from the surrounding bone was measured in Newton (N) at a speed of 1 mm/min.

2.9 | Serum analysis

The prior obtained blood samples were used to assess the bone metabolism as following: bone formation marker (Rat Osteocalcin ELISA Kit, Elabscience®) and bone resorption markers (Rat BMP-7 ELISA Kit, Elabscience® and Rat BMP-2 ELISA Kit, Elabscience®) (Shetty, Kapoor, Bondu, Thomas, & Paul, 2016). The entire procedure was performed according to the manufacturer's indications.

2.10 | Statistical analysis

The sample size and the statistical power were calculated before group allocation using StatMate® software. The sample size was calculated using the results obtained by previous studies on titanium osseointegration following alendronate and simvastatin administration, resulting in a total of 12 subjects per group (Duan, Ma, Li, Wang, & Liu, 2017; Ozaras & Rezvani, 2010; Shahrezaee et al., 2018). The type I/II error rates used for calculations were the alpha value of .05 and the power of 80%. Assuming a 20% mortality rate due to the surgical interventions and general anesthesia, three additional subjects per group were included (resulting in a total of 15 subjects per group), with a total of 75 subjects required in this study.

Statistical analysis was done using GraphPad Prism 6.0® software. The primary outcome measure was the assessment of tibolone impact on the osseointegration process using histological, micro-CT, mechanical, and serum markers analysis. The secondary outcome measure was to compare tibolone, alendronate, and simvastatin effect to control groups and between each other using the examinations mentioned above. We calculated means, standard deviations, frequencies, percentages, and correlation tests. Normal distribution was calculated using Shapiro–Wilk test and showed that the variables were normally distributed. To compare differences among groups, we calculated ANOVA with Bonferroni correction. The results were considered statistically significant if the adjusted *p*-value was less than .05.

We performed the study according to ARRIVE guidelines.

3 | RESULTS

During the study, a total of 12 rats died following the two surgical procedures (six rats in Group I, one rat in Group III, one rat in Group IV, and three rats in Group V), caused by anesthetic overdose (10 cases) and infection (two cases). We have also excluded six rats due to unsuccessful treatment administration (two in Group IV and four in Group V). During the autopsy, we found that all the ovariectomized rats had uterus atrophy and lack of ovaries, compared to a sham-ovariectomized group where the uterus was healthy, and the ovaries were in situ.

3.1 | Weight

At the beginning of the study, the average weight in ovariectomized animal models was 306 grams, compared to sham-ovariectomized rats, where the average was 256 grams (p-value < .01).

The average weight at the end of the study was 311 grams in Group I (OVX), 247 grams in Group II (sham-OVX), 308 grams in Group III (OVX + alendronate), 318 grams in Group IV (OVX + simvastatin), and 305 grams in Group V (OVX + tibolone). Statistical significant differences were between Group II (sham-OVX) and all of the other groups (p < .05).

The differences in weight at the beginning of the treatment and the weight at the end of the study are available in Table 1. The two statistically significant differences were found between Group I (OVX) and Group V (tibolone + OVX) (p = .035) and between Group IV (simvastatin + OVX) and Group V (tibolone + OVX) (p = .016).

3.2 | Micro-CT examination

The results of bone volume (BV), percent bone volume (BV%), bone surface (BS), tissue surface (TS), bone surface/volume ratio (BS/VR), mean total cross-sectional bone area, cross-sectional thickness, trabecular diameter, and trabecular number are represented in Table 1 and Figure 5, along with the statistically significant results between groups.

Regarding the peri-implant bone volume, the only statistically significant result was obtained between the OVX group and tibolone group (p = .004). Tibolone also had a statistically significant higher peri-implant percent bone volume and bone surface compared to OVX group (p = .004; p = .020). The bone surface/volume ratio (BS/VR) was higher in OVX group compared to sham-OVX, simvastatin, and tibolone groups (p = .001; p = .020; p = .001),

TABLE 1 Results of weight and micro-CT examinations expressed in mean (±standard deviation)

Examination	Parameter	Group I (OVX)	Group II (sham OVX)	Group III (alendronate + OVX)	Group IV (simvastatin + OVX)	Group V (tibolone + OVX)
Weight	Weight difference between treatment initiation and end of study (grams)	15.75[e] (15), *n* = 8	3.72 (24), *n* = 11	3.5 (21), *n* = 14	15.17[e] (15), *n* = 12	−0.66[a,d] (11), *n* = 8
Micro-CT analysis	Bone volume (BV)	0.17[e] (0.01), *n* = 3	0.33 (0.13), *n* = 3	0.27 (0.02), *n* = 4	0.29 (0.03), *n* = 4	0.40[a] (0.05), *n* = 4
	Percent bone volume (BV%)	1.78[e] (0.18), *n* = 3	3.41 (1.40), *n* = 3	2.82 (0.21), *n* = 4	3.05 (0.32), *n* = 4	4.10[a] (0.52), *n* = 4
	Bone surface (BS)	37.39[e] (5.27), *n* = 3	49.35 (17.34), *n* = 3	51.06 (1.9), *n* = 4	51.64 (1.93), *n* = 4	59.11[a] (4.38), *n* = 4
	Tissue surface (TS)	39.73 (2.21), *n* = 3	41.34 (1.10), *n* = 3	39.87 (1.59), *n* = 4	38.73 (1.28), *n* = 4	42.54 (2.36), *n* = 4
	Bone surface/volume ratio (BS/VR)	213.3[d,e] (16.01), *n* = 3	151.3 (18.75), *n* = 3	184.9[e] (11.52), *n* = 4	173.4[a] (12.25), *n* = 4	148.1[a,c] (13.2), *n* = 4
	Mean total cross-sectional bone area	0.02[e] (0.002), *n* = 3	0.041 (0.01), *n* = 3	0.034[a,e] (0.002), *n* = 4	0.036[a,e] (0.004), *n* = 4	0.049[a] (0.006), *n* = 4
	Cross-sectional thickness	0.010[b,e] (0.0001), *n* = 3	0.015[a] (0.001), *n* = 3	0.012[e] (0.001), *n* = 4	0.012[e] (0.0009), *n* = 4	0.016[a,c,d] (0.002), *n* = 4
	Trabecular diameter	0.018[b,e] (0.001), *n* = 3	0.026[a] (0.003), *n* = 3	0.021[e] (0.001), *n* = 4	0.023 (0.001), *n* = 4	0.027[a,c] (0.002), *n* = 4
	Trabecular number	8.03 (0.82), *n* = 3	7.65 (1.34), *n* = 3	8.75 (0.32), *n* = 4	8.51 (0.16), *n* = 4	8.41 (0.38), *n* = 4
Histology	Peri-implant compact bone width (μm)	30.58[b,c] (±8.43), *n* = 4	67.64[a] (±16.79), *n* = 11	66.67[a] (±26.21), *n* = 9	60.13 (±12.65), *n* = 8	69.50 (±19.43), *n* = 9
Mechanical pull-out test	F_{max} *(N)*	14.60 (±18.82), *n* = 5	47.32 (±42), *n* = 6	104.3 (±60), *n* = 5	74.13 (±61.41), *n* = 4	126 (±158), *n* = 6
Serum markers	Osteocalcin	13.99 (9.21), *n* = 7	26.86 (33.6), *n* = 9	36.41 (30.36), *n* = 10	31.06 (27.35), *n* = 12	38.0 (32.69), *n* = 7
	BMP-2	1,348 (663), *n* = 8	1,333 (321), *n* = 10	1,158 (341), *n* = 6	1,256 (356), *n* = 12	1,018 (404), *n* = 8
	BMP-7	277.7 (99), *n* = 7	333.8 (92), *n* = 10	253.3 (164), *n* = 7	280 (79), *n* = 13	239 (114), *n* = 8

[a]Statistically significant difference compared to Group I ($p < .05$);
[b]Statistically significant difference compared to Group II ($p < .05$);
[c]statistically significant difference compared to Group III ($p < .05$);
[d]statistically significant difference compared to Group IV ($p < .05$);
[e]statistically significant difference compared to Group V ($p < .05$).

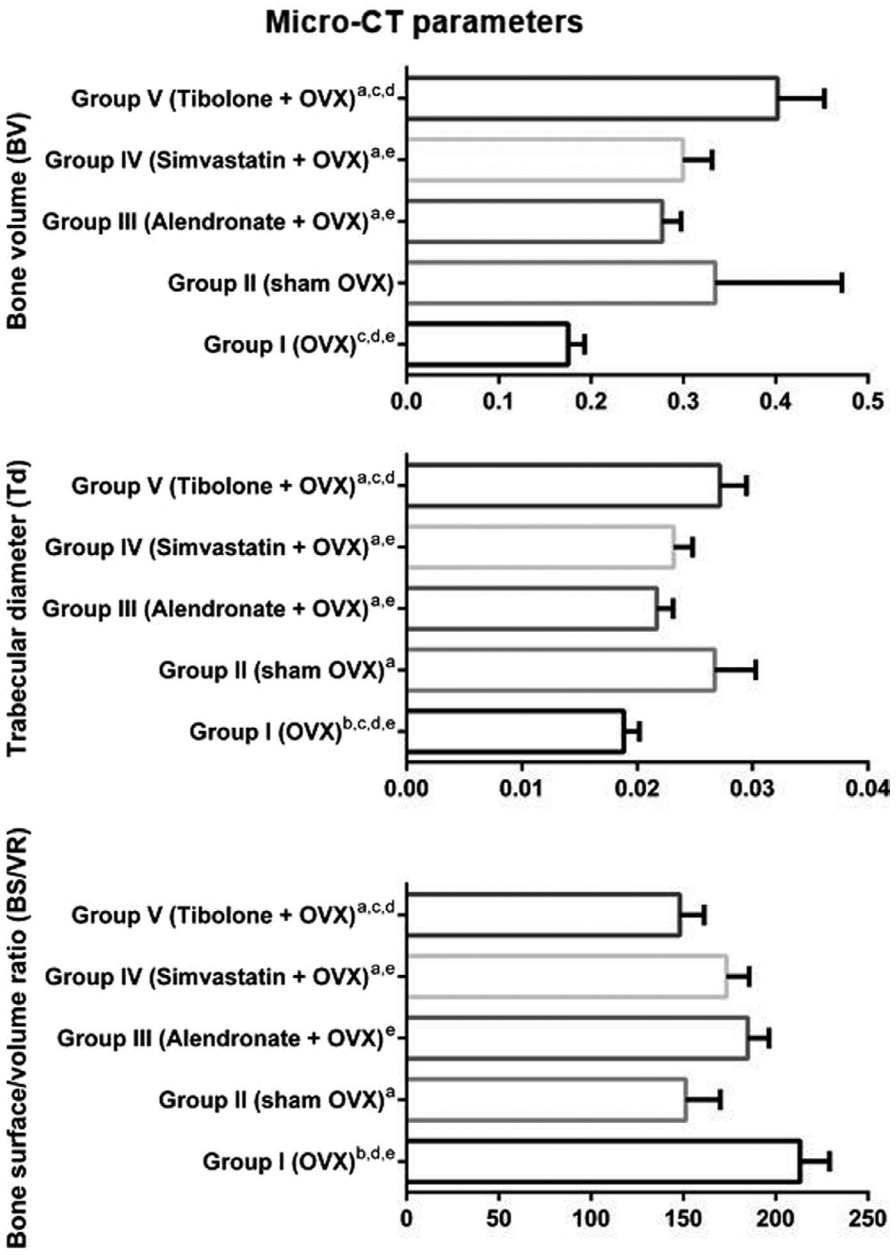


FIGURE 5 Boxplot of micro-CT examinations, including bone volume, trabecular diameter, and bone surface/volume ratio. [a]Statistically significant difference compared to Group I ($p < .05$); [b]Statistically significant difference compared to Group II ($p < .05$); [c]Statistically significant difference compared to Group III ($p < .05$); [d]Statistically significant difference compared to Group IV ($p < .05$); [e]Statistically significant difference compared to Group V ($p < .05$)

while alendronate had a statistically significant ratio compared to tibolone ($p = .020$). The mean total cross-sectional bone area was higher in tibolone group compared to OVX group ($p = .004$). In terms of cross-sectional thickness, the statistically significant results were higher in tibolone group compared to OVX, alendronate, and simvastatin groups ($p = .001$; $p = .010$; $p = .030$). Moreover, the OVX group had a higher cross-sectional thickness compared to sham OVX ($p = .007$). The trabecular diameter was higher in tibolone group compared to OVX and alendronate groups ($p = .002$; $p = .030$), while OVX group had a lower value compared to sham OVX ($p = .005$).

Longitudinal images of the titanium implant obtained during micro-CT examination are available in Figure 6. Group I had bone bridges (arrow, Figure 6a) that link the implant to the femoral cortical bone. Group II had thicker bone bridges (arrow, Figure 6b) compared to Group I, and it also presents a visible bone layer surrounding the implant (arrowhead, Figure 6b). Alendronate group did not show bone bridges in the examined specimens ($n = 4$), but the implant was stabilized distally by a more abundant trabecular bone (arrow, Figure 6c). Simvastatin presented bone bridges proximally (arrow, Figure 6d) and a more visible bone tissue surrounding the implant compared to groups I, II, and III (arrowhead, Figure 6d). Tibolone group implants, in addition to bone bridges (arrow, Figure 6e), were surrounded by a thicker and more consistent bone tissue compared to any other group (arrowhead, Figure 6e).

Results of bone mineral density are available in Figure 7a. Bone mineral density was significantly different among groups. The statistically significant results were as follows: Group I (OVX) versus Group II (sham-OVX) ($p = .028$); Group I (OVX) versus Group III (alendronate + OVX) ($p = .006$); Group I (OVX) versus Group V

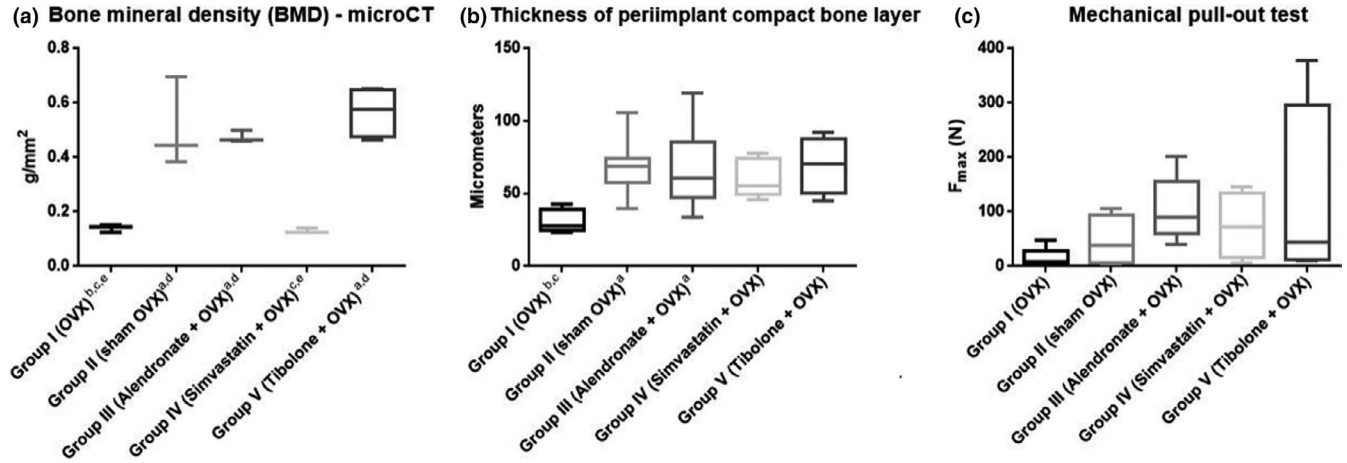


FIGURE 6 Longitudinal micro-CT images of specimens: (a) Group I (OVX); (b) Group II (sham-OVX); (c) Group III (alendronate + OVX); (d) Group IV (simvastatin + OVX); (e) Group V (tibolone + OVX)

(tibolone + OVX) (p = .001); Group II (sham-OVX) versus Group IV (simvastatin + OVX) (p = .002); Group III (alendronate + OVX) versus Group IV (simvastatin + OVX) (p = .005); and Group IV (simvastatin + OVX) versus Group V (tibolone + OVX) (p = .001).

Results of bone–implant contact are represented in Figure 8. The statistically significant results were as follows: Group I (OVX) versus Group III (alendronate + OVX) (p-value = .043); Group I (OVX) versus Group IV (simvastatin + OVX) (p-value = .009); Group III (alendronate + OVX) versus Group IV (simvastatin + OVX) (p-value = .030); and Group IV (simvastatin + OVX) versus Group V (tibolone + OVX) (p-value = .008).

3.3 | Histological analysis

In all groups, the intramedullary titanium implants had a surrounding compact bone and periosteal layer in direct contact with the implant (Figure 9a). The peri-implant bone tissue was separated

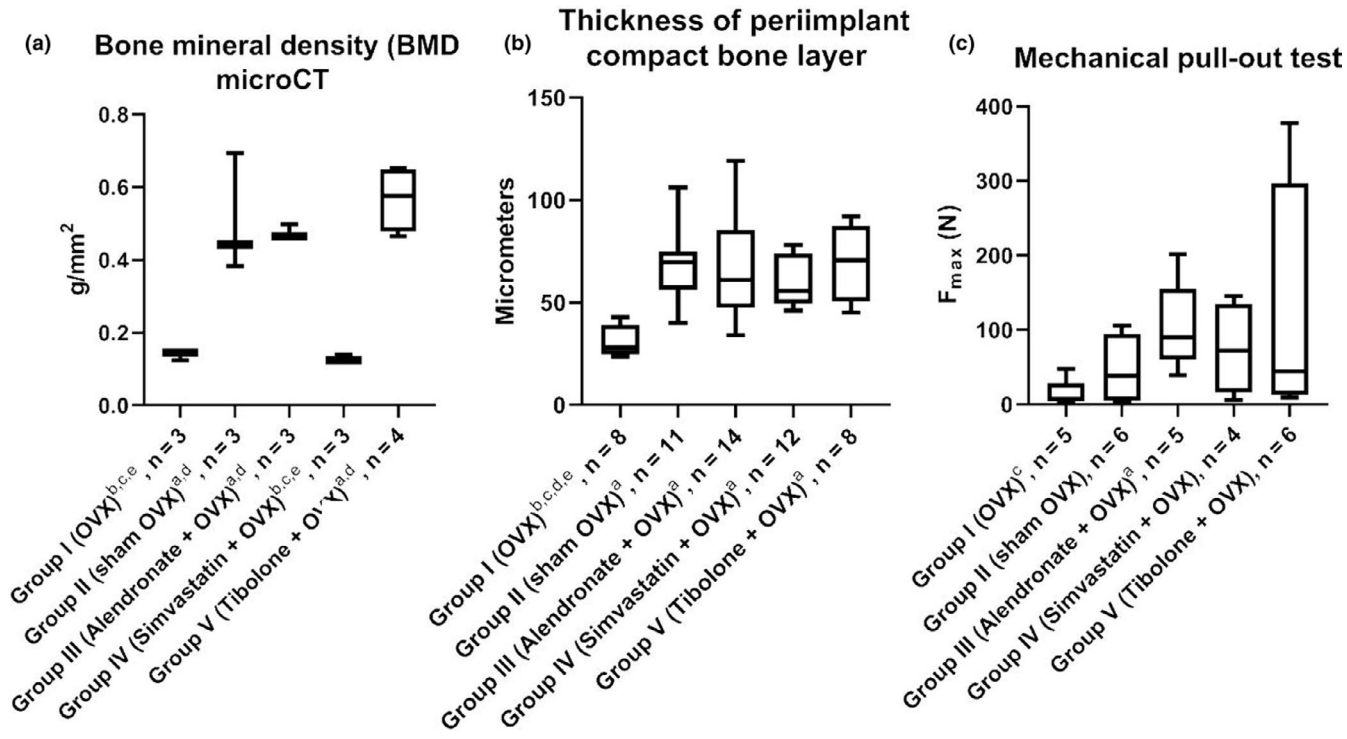


FIGURE 7 Boxplot of (a) bone mineral density determined using micro-CT examinations; (b) compact bone surrounding the implant; (c) mechanical pull-out test; [a]Statistically significant difference compared to Group I (p < .05); [b]Statistically significant difference compared to Group II (p < .05); [c]Statistically significant difference compared to Group III (p < .05); [d]Statistically significant difference compared to Group IV (p < .05); [e]Statistically significant difference compared to Group V (p < .05)

from the compact femoral bone by bone marrow (Figure 9b). Moreover, perpendicular bone lamellae had bridged the newly formed compact bone layer surrounding the implant to the cortical bone (Figure 9c). These bridges were more frequent in epiphyseal and metaphysical regions compared to the bone diaphysis. Examples of the peri-implant cortical bone layer in each group are available in Figure 10a-e, and the peri-implant compact bone layer thickness results are available in Figure 9b. Statistically significant differences were obtained between Group I (OVX) and Group II (p = .018), as well as between Group I (OVX) and Group III (alendronate + OVX) (p = .030), while no statistically significant differences were obtained between sham-OVX and treatment groups.

3.4 | Mechanical pull-out test

The mean maximum forces (F_{max}) in Newtons (N) are available in Figure 7c. Forces of over 200 N were obtained in two cases belonging to the tibolone group (269 N and 377 N) and in one case of alendronate group (201 N). Although the highest average was obtained in the tibolone group, a heterogenic distribution of values was also acquired [min = 9.04 N, max = 377 N, SD = ±158, n = 6]. There were no statistically significant results between groups.

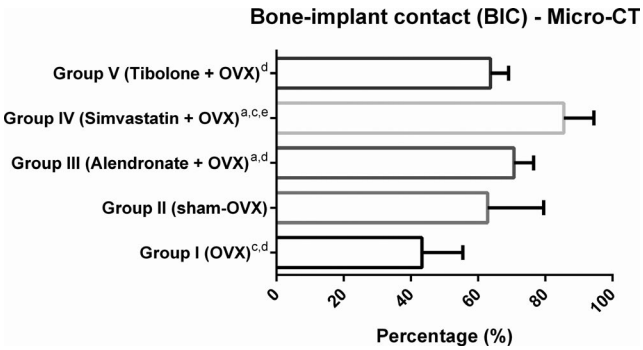


FIGURE 8 Boxplot of peri-implant bone formation obtained during micro-CT examinations. [a]Statistically significant difference compared to Group I (p < .05); [b]Statistically significant difference compared to Group II (p < .05); [c]Statistically significant difference compared to Group III (p < .05); [d]Statistically significant difference compared to Group IV (p < .05); [e]Statistically significant difference compared to Group V (p < .05)

3.5 | Serum analysis

Results of serum markers determination, including osteocalcin, BMP-2, and BMP-7, are found in Figure 11. Although no statistically significant results were obtained among groups, tibolone had almost three times more quantity of bone formation marker osteocalcin compared to the OVX group and was closely followed by alendronate. All of the treatment groups showed a better bone formation compared to OVX and sham-OVX groups, but the results were not statistically significant.

4 | DISCUSSIONS

To our knowledge, this is the first study to compare alendronate and simvastatin, two commonly used drugs shown to improve the osseointegration of titanium implants (Apostu et al., 2017). Furthermore, it is the first study to introduce a new class of drugs, represented by tibolone, in the field of osseointegration.

Due to a high resemblance between rat bone metabolism and human bone metabolism, we used rat animal model which is most commonly used in the current literature for the study of implant osseointegration process (Alghamdi, van den Beucken, & Jansen, 2014; Apostu et al., 2017; Back et al., 2012; Du et al., 2009). An ovariectomized animal model had been selected for the induction of osteoporosis because most previous studies were performed on ovariectomized rats; therefore, this allows for a comparison among our results and previous results obtained in the literature (Apostu et al., 2017). The bone formed around the implants under the effect of systemic drugs can be affected by the osteoporotic condition, but this has a better resemblance to clinical situations where patients are frequently osteoporotic and have a deficient bone metabolism (da Silva Mello, et al., 2015). Previous studies showed that in the case of osteoporotic conditions, the impact of different agents is higher, resulting in the need of fewer subjects to obtain statistically significant results (Oh KC, Moon, Lee, Park,

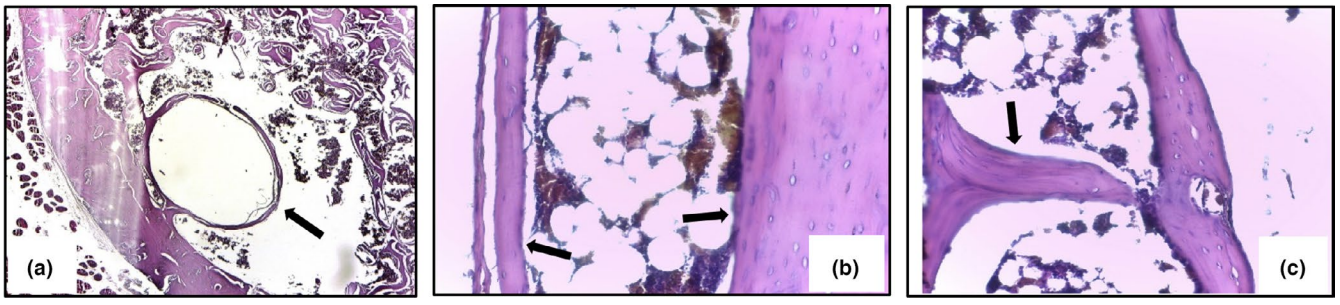


FIGURE 9 Histological images in hematoxylin–eosin staining: (a) bone layer surrounding the implant situ (arrow) at ×100 magnification; (b) bone-periosteal layer (left arrow) and compact femoral bone (right arrow) separated by bone marrow (×200 magnification); (c) bone lamellae perpendicular which anchors the peri-implant bone layer to compact femoral bone (arrow) (×200 magnification)

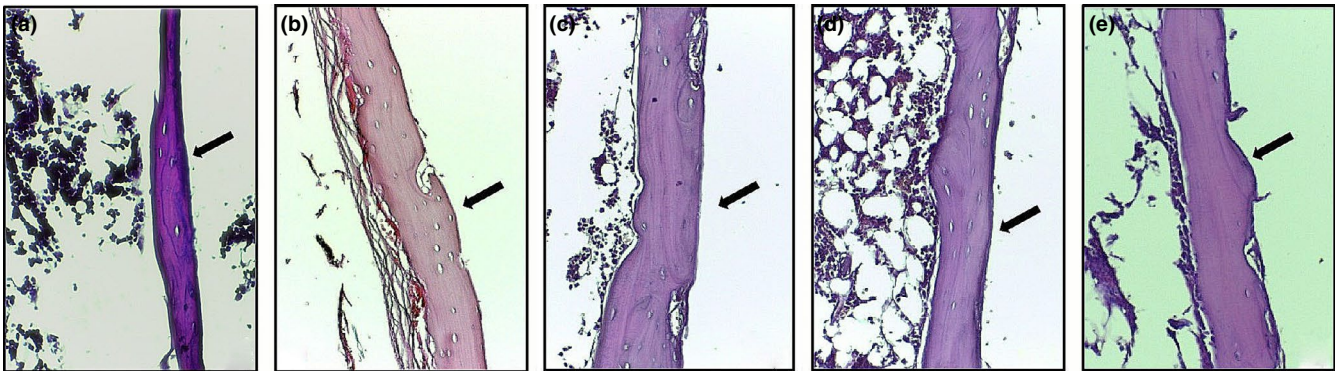


FIGURE 10 Representation of the cortical bone layer (arrow) formed around the titanium implant in hematoxylin–eosin staining (200× magnification). (a) Group I (OVX); (b) Group II (sham-OVX); (c) Group III (alendronate + OVX); (d) Group IV (simvastatin + OVX); (e) Group V (tibolone + OVX)

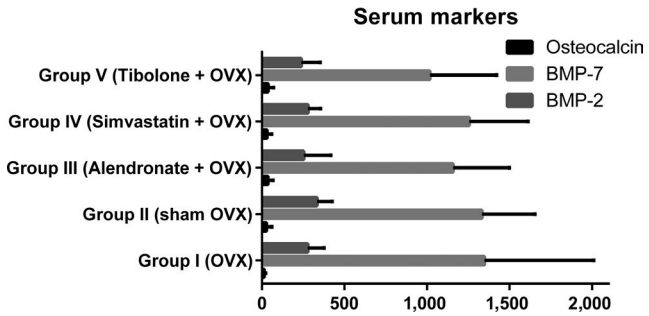


FIGURE 11 Boxplot of serum examinations, including osteocalcin, BMP-2, and BMP-7. [a]Statistically significant difference compared to Group I ($p < .05$); [b]Statistically significant difference compared to Group II ($p < .05$); [c]Statistically significant difference compared to Group III ($p < .05$); [d]Statistically significant difference compared to Group IV ($p < .05$); [e]Statistically significant difference compared to Group V ($p < .05$)

& Kim, 2015; Viera-Negron, et al., 2008). There is no clear evidence in the current literature on when the osteoporosis following the ovariectomy procedure is fully developed, for a proper initiation of treatment, but previous studies showed that more significant differences are obtained at 12 weeks postoperatively (Gao, Ma, Dong, Yong, & Su, 2014; Lei, Yuan, & Gao, 2017). This was the reason why our study group decided to initiate the intramedullary implantation and treatment 12 weeks after ovariectomy. Although we did not perform a BMD examination before the intramedullary nail procedure, the weight in the sham-OVX was significantly reduced compared to ovariectomized rats before implant intervention. Also, the BMD showed significant a reduction in the case of ovariectomized rats compared to sham-OVX, meaning a successful induction in osteoporosis.

In our study, titanium nail implants were used instead of screw implants as most studies involving bisphosphonates in the literature were performed on titanium nails (Kellesarian et al., 2017). Therefore, we could compare our results to a higher number of studies in the current literature.

We decided to end the study 3 months following the implantation of titanium implants because previous studies showed that at this period, the morphological and biochemical changes within the osseointegration process are reduced to the minimum (Bottai et al., 2015). Earlier assessment of the osseointegration process can lead to results that do not resemble the final output.

Although simvastatin proved superior effects on the process of osseointegration when applied locally in the oral cavity, we decided to use systemic administration in our study for a better comparison with the effects of alendronate and tibolone (Gupta, Fabbro, & Chang, 2019). The alendronate and simvastatin treatment doses used in our study are similar to the ones previously described in the literature, while in case of tibolone treatment, we used a dose shown to have a positive impact on bone metabolism (Carvalho et al., 2012; Du et al., 2009; Lei et al., 2009; Vohra, Al-Rifayi, Almas, & Javed, 2014; Zhang, Tian, & Luo, 2010; Zhou et al., 2018). Regarding treatment administration, difficulties in the oral administration of tibolone were encountered, most probably due to the organoleptic properties of tibolone.

The results regarding the weight are according to other studies in the literature. Ovariectomized rats gained in weight compared to sham-ovariectomized rats, while simvastatin administration did not influence the weight gain in ovariectomized rats (Hata, 2009). Although no statistically significant, alendronate protected against excessive weight gain in ovariectomized rats in our study, as supported by the literature (Chen et al., 2014). On the other hand, tibolone administration limited the weight gain in our study, similar to a previous study (da Silva Mello et al., 2016).

Alendronate and simvastatin treatments had both showed an improvement in the osseointegration process in case of histological, imagistic, and mechanical examinations compared to ovariectomized rats, results supported by other studies in the literature, but due to the limited number of subjects, the only statistically significant results were regarding the mean total cross-sectional bone area (alendronate and simvastatin), bone surface/volume ratio (simvastatin), and thickness of the peri-implant bone layer (alendronate) (Apostu et al., 2017; Chen et al., 2013; Oliveira et al., 2017; Park et al., 2013). In our study, alendronate had superior results to simvastatin in terms of histological measurement of peri-implant bone width and mechanical pull-out test, but the differences were not statistically

significant due to a relatively low number of subjects. Regarding micro-CT analysis, the results were similar except for BMD assessment, which was statistically significant in the alendronate group compared to simvastatin. The result was expected, as alendronate is a bisphosphonate, which is mainly indicated in osteoporosis prevention. Simvastatin, on the other hand, is a lipid-lowering agent with no current indication in the treatment of osteoporosis. Longitudinal micro-CT images showed that implant fixation was predominantly in the distal femur for alendronate group due to a better trabecular bone at that level, while in the simvastatin group, the fixation was better in the proximal femur due to bone bridges. Moreover, simvastatin showed a higher content of bone layer surrounding the implant compared to alendronate. All of these results indicate that alendronate had a centripetal implant fixation (from host bone tissue to the implant), while simvastatin had a more centrifugal osseointegration process (from implant to host bone tissue).

Peri-implant bone formation can be calculated using micro-CT examinations, although the term is mostly used in case of histological examinations and it is gaining more importance due to the advantage of being non-invasive (Bernhardt, Kuhlisch, Schulz, Eckelt, & Stadlinger, 2012; Bissinger et al., 2017; Choi et al., 2018; Jimbo et al., 2011; Stadlinger et al., 2013). Moreover, the peri-implant bone formation calculated based on micro-CT images is similar to the one calculated in histological examinations (Bissinger et al., 2017; Jimbo et al., 2011). Simvastatin treatment showed more coverage of the implant by bone tissue compared to other groups, followed by alendronate and tibolone. We found that the superior results of the simvastatin group regarding peri-implant bone formation did not correlate with superior results in the mechanical pull-out test. We consider that this can be explained by the fact that although the percentage of implant surface which is in contact with bone tissue, represented by peri-implant bone formation, is an essential factor for implant stability, other peri-implant parameters which quantify the bone quantity such as bone volume (BV) can also play an essential role in the strength of implant fixation.

Considering that alendronate is administered twice a week compared to simvastatin, which is administered daily, the adherence rate for alendronate is expected to be higher in clinical situations. Alendronate, contrary to simvastatin, also protected against weight gain following osteoporosis. Therefore, we consider that alendronate is more appropriate for future clinical trials. On the other hand, alendronate is expected to have more common side effects such as bone, muscle pain, joint pain, or even the pathological fractures described following the use of bisphosphonates, so simvastatin can represent a good alternative in higher-risk patients (Ozaras & Rezvani, 2010; Saita, Ishijima, & Kaneki, 2015).

As previously stated, alendronate and simvastatin have two different mechanisms of action: anti-catabolic action in case of alendronate and dual anabolic anti-catabolic mechanism in case of simvastatin (Apostu et al., 2017). As alendronate was slightly superior to simvastatin in the examinations performed within our study, it indicates that the anti-catabolic actions have the most critical role throughout the osseointegration process.

As mentioned before, tibolone has never been studied before in the osseointegration process. It is currently used in the treatment of postmenopausal osteoporosis where it increases spine and hip BMD (Lazovic et al., 2007). In our study, tibolone had proved to be an excellent agent in preventing the deficient osseointegration usually found in osteoporotic animal model. Tibolone had shown better results compared to alendronate, simvastatin, and sham-ovariectomized in the histological measurement of peri-implant bone width, mechanical pull-out test, and micro-CT analysis. Due to the relatively low number of subjects, the only differences found statistically significant were in micro-CT parameters. Bone surface/volume ratio around titanium implant, as well as trabecular diameter, was better in tibolone group compared to alendronate, while the cross-sectional thickness was higher in tibolone group compared to alendronate and simvastatin.

Nevertheless, the high bone mineral density could potentially lead to the same pathological fractures described in bisphosphonate use (Mathonet, Willems, & Ciornohac, 2018; Saita et al., 2015). On longitudinal micro-CT images, tibolone showed a better bone tissue layer around the implant compared to any of the treatment groups, and it presented the highest values in the mechanical pull-out test, but the results were not clustered, as in case of alendronate, which offered more consistent results. Tibolone group was the only case where no weight gain was achieved following the treatment. We have no clear explanation for this result, as the rats were still undergrowth; therefore, a small increase in weight should have been obtained. Overall, the results showed a possible variable effect of tibolone on the osseointegration process, but more studies are needed to confirm these results. Considering that previous studies showed that tibolone did not increase the risk of long-term adverse events, this drug represents a right candidate for postoperative enhancement of orthopedic titanium implants osseointegration (Formoso et al., 2016).

The blood serum markers assessed in the present study were osteocalcin, BMP-2, and BMP-7. Osteocalcin is a specific biomarker for osteoblast function, frequently used in experimental and clinical settings (Kuo & Chen, 2017). BMP-2 and BMP-7 are growth factors that induce the differentiation of mesenchymal stem cells into osteoblasts and promote their proliferation (Yang et al., 2014). We aimed to assess the bone formation processes using osteocalcin marker and to determine whether the induced osteoporosis or treatment with alendronate, simvastatin, and tibolone can influence the BMP-induced osteoblastogenesis. Although no statistically significant differences had been obtained among serum markers due to a limited number of tests, tibolone and alendronate groups had almost three times the osteocalcin level of the control group. This shows a more important anabolic effect of these agents on bone metabolism. Simvastatin and sham-OVX groups also showed an increase in osteocalcin compared to the OVX group, but a lesser extent.

In our opinion, the lack of significant differences in the case of BMP-2 and BMP-7 among groups is due to the reduction of the growth factors induced implant osseointegration process at the

time when the serum was obtained (Bottai et al., 2015; Jimi, Hirata, Shin, Yamazaki, & Fukushima, 2010; Spiro et al., 2010). We consider that if bone serum markers had been assessed earlier following the implantation, the differences would have been statistically significant. These results are according to other studies in the literature (Altundal & Gursoy, 2005; Ederveen & Kloosterboer, 2001; Xu et al., 2014). Contrary to our study, simvastatin and alendronate had been shown to increase BMP-2 levels in other studies, but this effect was obtained at a maximum 4 weeks after initiation of treatment (Çakır-Özkan et al., 2017; Liu, Yuan, & Gao, 2017). Correlated with the fact that the bone formation marker osteocalcin had higher levels in the treatment groups, we consider that the induction of anabolic bone process had ended in the treatment groups due to an accelerated initiation compared to Group I (OXV).

A significant limitation of our study was represented by the lack of non-decalcified implant-preserving histological analysis to evaluate the histological bone–implant contact (BIC). As micro-CT and pull-out mechanical tests have proved their ability to analyze the osseointegration process, along with the information about cortical bone surrounding the implant, obtained from decalcified samples, we consider that the lack of non-decalcified samples, although adding valuable information, did not influence the result of our study (He et al., 2017; Jung-Yoo, In-Sung, Ji, & Jae-Il, 2019; Stadlinger et al., 2013). The limitations of our study include a relatively low number of subjects, not performing osteoporosis tests before the implantation procedure, lack of bone serum markers assessment throughout the osseointegration process, lack of pharmacodynamic analysis, and lack of quantification of drug levels in the body. The results should be interpreted with caution as the number of samples calculated during the study design was not achieved for all analysis. Another limitation of the animal model was represented by a complicated administration of oral drugs mixed with food, as gavage could not be used in this study for obtaining drug administration in all cases due to an unacceptably high risk of oesophageal perforation in long-term treatment (Office for Research Ethics & Integrity, 2018; Zhang et al., 2010).

5 | CONCLUSIONS

The hypothesis that alendronate, simvastatin, and tibolone enhance the osseointegration process of intramedullary titanium implants in ovariectomized rats has been accepted. Tibolone group showed a slightly better osseointegration process based on micro-CT results in our study compared to alendronate and simvastatin. No statistically significant results were obtained between treatment groups in terms of histological analysis and mechanical pull-out tests. In addition to simvastatin, alendronate and tibolone protected against bone mineral density (BMD) loss and weight gain following ovariectomy. Bone serum markers were not statistically significantly different between groups.

The present study proved that systemic drugs such as alendronate, simvastatin, and tibolone could improve the osseointegration rate of titanium implants, with minimum cost and without creating any additional burden for the patient.

6 | ETHICAL REVIEW COMMITTEE STATEMENT

The study received the Ethics Commission approval (no. 467/14.12.2016) as well as the Sanitary Veterinary and Food Safety Agency approval (no. 33/06.02.2017). The experiments were performed at the Centre of Experimental Medicine Cluj-Napoca, according to ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments.

The housing of animal models, surgical procedures, treatment administration, and micro-CT examinations were performed at the Department of Animal Facility within the Iuliu Hatieganu University of Medicine Cluj-Napoca. The mechanical pull-out test was performed at the Department of Materials Science and Engineering of Cluj-Napoca Technical University. The serum laboratory tests were performed at Prof. Dr. Octavian Fodor Regional Institute of Gastroenterology and Hepatology Cluj-Napoca.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Alexandru Mester and Daniel Oltean-Dan have contributed equally to this manuscript as the first author. D.A., O.L., D.O.D., A.M., H.R.C.B., and C.B. participated in the study design; D.A., O.L., D.O.D., and A.M. performed the experiments; D.A. and D.G. collected the data; D.A., H.R.C.B., and O.L. took part in data analysis; D.A., O.L., H.R.C.B., D.O.D., D.G., and A.M. interpreted the data; and D.A., O.L., H.R.C.B., and A.M. contributed to manuscript preparation. All authors revised the manuscript and approved its final version.

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