Effects of Chronic Stress and Alendronate Therapy on the Osseointegration of Titanium Implants

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ABSTRACT

Purposes: The purposes of this study were to evaluate the influence of chronic stress (CS) on implant osseointegration and also to analyze whether alendronate (ALN) therapy could prevent these eventual stress-negative effects.

Materials and Methods: Adult male Holtzmann rats were assigned to one of the four experimental groups: AL (ALN; 1 mg/kg/week; $n = 12$), ALS (ALN + CS; 1 mg/kg/week; $n = 12$), CTL (sterile physiological saline; $n = 12$), or CTLS (sterile physiological saline + CS; $n = 12$). After 58 days of drug therapy, the ALS and CTLS groups were exposed to CS, and 2 days later all animals underwent tibial implant installation. The animals were euthanized 28 days following the operative surgical procedure.

Results: It was observed that the CTLS group presented an impairment of bone metabolism represented by lowest levels of bone-specific alkaline phosphatase and bone area fraction occupancy values. Furthermore, these animals presented a higher proportion of empty osteocytic lacunae. In contrast, the ALN therapy showed increased osseointegration and torque value parameters, regardless of stress exposition.

Conclusions: Analysis of the data presented suggests that CS partially impairs the osseointegration of tibial implants and that ALN therapy is able to prevent these negative effects.

KEY WORDS: alendronate, bone, operative surgical procedure, osseointegration

INTRODUCTION

The relationship between stress and health has been the focus of many studies over the years. Indeed, a substantial body of evidence confirms that stress may play a relevant role in the pathogenesis of several diseases[1–4] and may also have been associated with the impairment of soft tissue wound healing.[5–7]

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Stressful events activate the hypothalamic-pituitary-adrenal (HPA) axis and increase the release of corticotrophin-releasing hormone from the hypothalamic paraventricular nucleus, causing the secretion of adrenocorticotropin from the anterior pituitary, which stimulates the secretion of corticosterone from the adrenal cortex.[8,9]

Although the mechanisms responsible for stress-induced impairment of wound healing have not yet been totally elucidated, evidence suggests that the most prominent pathway is through activation of the HPA axis.[10] Indeed, in vivo studies showed that the administration of glucocorticoids (GCs) delays soft tissue wound healing[11] and impairs implant osseointegration.[12]

In the field of bone metabolism, many efforts have been made to develop strategies to stabilize the bone loss occasioned by diseases and drugs. In this context, bisphosphonates (BPs) have demonstrated positive effects on bone tissue, improving the osseointegration of

implants[13–15] and preventing bone loss induced by the administration of GC.[12,16]

Although there is substantial evidence that exposure to chronic stress (CS) might lead to wound-healing impairment, the effects of stress on the osseointegration process have not yet been investigated. Therefore, the purposes of this study were to evaluate the influence of CS on osseointegration parameters and to analyze whether alendronate (ALN) therapy can prevent these eventual stress-negative effects.

MATERIALS AND METHODS

Animals

The study was approved by the Ethics in Animal Research Committee of the School of Dentistry of Araraquara (UNESP, Araraquara, Brazil) (protocol number 18/2009) and included a total of 48 male Holtzmann Albino rats, each weighing around 220 g. These animals were housed individually in single cages and kept in a special facility room at São Paulo State University – UNESP, School of Dentistry of Araraquara. Rats were maintained on a 12:12-hour light/dark cycle (lights on at 7:00 a.m.) at 23 ± 2°C, with ad libitum access to a standard laboratory diet and water. Body weight was monitored regularly.

Drugs

The ALN was purchased from ALCON Laboratory (São Paulo, SP, Brazil). The drug was dissolved in sterile physiological saline (0.9% NaCl) and diluted to the given concentration.

Experimental Protocol (Figure 1)

After a 3-day acclimatization period, animals were randomly assigned to one of the four experimental groups: AL ($n = 12$) and CTL (control; $n = 12$) groups, including animals treated with subcutaneous administration of 1 mg/kg of ALN[17,18] or sterile physiological saline once a week, respectively, or ALS (ALN + stress; $n = 12$) and CTLS (control + stress; $n = 12$) groups, comprised of animals subjected to CS and treated with ALN or sterile physiological saline, respectively, following the same posology of nonstressed groups.

Implant Surgical Procedure

After 60 days of ALN or sterile physiological saline treatment, all animals were anesthetized by a combination of ketamine hydrochloride (Ketamina Agener, Agener União Ltda, São Paulo, SP, Brazil; 0.08 mL/100 g body weight) and xylazine 2% (Rompum, Bayer S.A., São Paulo, SP, Brazil; 0.04 mL/100 g body weight) and underwent trichotomy on the inner region of the leg and asepsis with povidone iodine solution. After that, an incision was made in the layers of the tibial metaphysis. The underlying bone was subjected to osteotomy with a starting drill of 1.8 mm to accommodate the machined titanium implant with 4-mm length and 2.2-mm diameter (Neodent, Curitiba, Brazil) under abundant irrigation. The tissue was sutured with 4-0 silk thread (Ethicon, Division of Johnson & Johnson Medical Limited, São Jose dos Campos, São Paulo, Brazil).

Postoperatively, all animals received an intramuscular dose of antibiotic (Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, Brazil – 0.1 mg/kg) and anti-inflammatory Ketoflex (Ketoprofen 1.0%, 0.03 mL/rat). The ALN or saline solution administration was maintained until 28 days after surgical procedures, when all animals were euthanized by anesthesia overdose (administration of ketamine and xylazine intraperitoneally at three times the anesthetic dose).

Stress Paradigm

The CS protocol was adapted from Marin and colleagues,[19] beginning 2 days before the surgical procedures. The protocol consisted of exposure to three different cycles of stressors (10 consecutive days, each

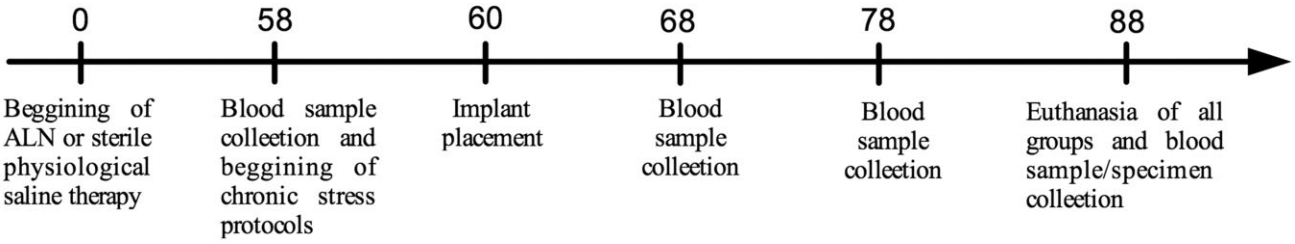

Figure 1 Experimental design. ALN, alendronate.

cycle) once a day for 30 days. In the first cycle, rats were housed singly in plastic cages without bedding in a cold chamber at 4°C, 15 min/day. The cold chamber consisted of a commercial fridge with ventilation cooling system and electronic temperature controller. In the second cycle, the rats were restrained in plastic cylinders (20.0 cm [length] × 5.5 cm [internal diameter]) for 1 h/day. In the last cycle, they were subjected to forced swimming for 4 min/day.

All the stress sessions were performed in a room adjacent to the animal facility, and the nonstress group was left undisturbed except when their cages were cleaned.

Histological Analysis

Initially, all tissue blocks were immersed directly in 10% buffered formalin fixative solution for 48 hours. After that, six specimens of each group underwent routine histological processing, while six other specimens were prepared for hard tissue histology.

Routine Histology

In this analysis, all specimens were decalcified in tetrasodium-ethylenediaminetetraacetic acid aqueous solution (0.5 M, pH 7.4) for 2 to 3 months under agitation at room temperature. After that, the tibial implants were carefully removed, processed, and included in paraffin blocks. Serial 4-μm sections were obtained in the buccolingual direction, stained with hematoxylin and eosin, and referred for light microscopy (Leica DM1200M; Leica Microsystems, Wetzlar, Hesse, Germany) for descriptive and quantitative evaluation.

One board-certified oral pathologist blinded to the group assignments performed these analyses in three distinct moments to minimize discrepancies in the scores (kappa index = 0.76). The histological end points were evaluated at the lateral wall and the base part of the implant cavities[20] using magnifications of ×100, ×200, ×400, and ×1000. This analysis included the degree of empty osteocytic lacunae and inflammation that were scored on a four-point scale: 0 (absent; 0%), 1 (mild; ≤10%), 2 (moderate; >10 and ≤50%), and 3 (increased; >50%).

Hard Tissue Histology

In this analysis, the specimens containing implants were prepared after dehydration by a series of ethanol solutions and embedded in methacrylate-based resin (Technovit 7200; Heraeus Kulzer, Wehrheim, Hesse, Germany). The blocks were initially sectioned at about 150 μm using a specific system (EXAKT Apparatebau GmbH & Co., Norderstedt, Germany)[21] and subjected to grinding and polishing (EXAKT Apparatebau GmbH & Co.) to achieve a final thickness of approximately 30 μm. After that, the sections were stained with Stevenel's blue/acid fuchsin (1%) and referred for light microscopic evaluation.

Measurements of the percentages of bone-implant contact (BIC) and bone area fraction occupancy (BAFO) were performed at ×100 magnification (Leica DM1200M; Leica Microsystems) using ImageJ 1.410 (National Institutes of Health, Bethesda, MD, USA).

Torque Removal Analysis

Immediately after the animals were sacrificed, torque removal analysis was undertaken in six specimens from each group. The tibial implant was attached to a torque meter with a scale range of 3 to 30 Ncm and divisions of 0.05 Ncm (Tohnichi, Shanghai, China). [Correction made March 8, 2013 after online publication: scale range corrected.] A wrench was attached to the implant head to apply torque in the reverse direction of implant placement, until complete rupture of the bone-implant interface occurred, signaled by the rotation of the implant.

Assessment of Bone Turnover Biochemical Markers

Blood samples were collected on the day of sacrifice by cardiac puncture, and the serum was obtained after blood centrifugation at 3000 *g* for 10 minutes at 4°C and stored at −80°C until the analysis. The levels of serum collagen type 1 cross-linked C-telopeptide (CTX) and bone-specific alkaline phosphatase (BALP) (Cusabio Biotech Co., Ltd., Wuhan, People's Republic of China) were determined by enzyme-linked immunosorbent assay kits.

Radioimmunoassay of Corticosterone

Blood samples were collected four times during the experiment, previous to the first stress session (baseline) and 24 hours after each cycle of stressors, to evaluate the basal levels of corticosterone during stress exposition. The blood was collected from the rat caudal artery and immediately centrifuged at 3000 *g* for 10 minutes at 4°C and stored at −80°C until the analysis. The radioimmunoassay for corticosterone was conducted with antibody obtained from Sigma (St. Louis, MO, USA)

and (3H)-corticosterone from New England Nuclear (Boston, MA, USA). The method was adapted from that described by Sarnyai and colleagues.[22]

Statistical Analysis

The data were evaluated by means of the GraphPad Prism 5.0 software package (GraphPad Inc., San Diego, CA, USA). The normality of the data was assessed by the Kolmogorov-Smirnov test. The difference between the groups for parametric data was evaluated by analysis of variance followed by Tukey's tests or the *F*-test. Statistical significance was set at 5%.

RESULTS

Body Weight

The mean body weight of all rats was ~160 g at the beginning of the study, and there were no statistically significant differences among the groups ($p > .05$). During the period of restraint stress, while control groups exhibited an increase of body weight, the stressed animals presented a significant weight loss ($p < .001$) (Figure 2). In other cycles of stress, no statistical differences were found.

Routine Histology

Observations of histological sections showed that the bone tissue surrounding tibial implants in ALN groups was markedly compact, while a high volume of spongious and marrow bone was observed in control animals (Figure 3). Although empty osteocytic lacunae areas were observed in all the groups, animals in the ALS group presented the highest values, which were statistically different from those of the CTL animals ($p < .01$) (Figure 4A).

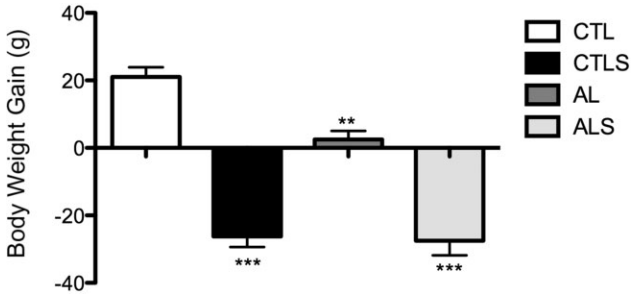

Figure 2 Body weight gain of rats subjected to repeated restraint stress. Data shown as mean ± SD; ***$p < .001$ in relation to CTL and AL; **$p < .01$ in relation to CTL. AL, alendronate; ALS, alendronate + stress; CTL, control; CTLS, control + stress.

Furthermore, in animals with no ALN treatment, the exposure to stressors was associated with a significant increase in empty osteocytic lacunae regions compared with that in the CTL group ($p < .05$) (Figure 4A). In contrast, animals in the ALS groups presented a higher degree of inflammation compared with those in the AL group ($p < .05$) (Figure 4B).

Hard Tissue Histology

At 28 days following implantation, all groups presented osseointegrated implants (Figure 3), and BAFO measurements revealed that the CTLS group presented the lowest values when compared with CTL- ($p < .05$) and ALN-treated animals ($p < .001$). Furthermore, animals of the AL group showed no statistical differences in relation to ALS and CTL (Figure 5A).

Regarding the BIC analysis, it was observed that animals treated with ALN presented the highest values compared with those in the control groups ($p < .001$). No significant differences were observed between the groups with and without stress in the BIC parameter, although control animals subjected to CS presented lower values when compared with CTL animals (Figure 5B).

Torque Removal Analysis

ALN treatment showed a significant increase in the torque removal values when compared with that in animals receiving sterile physiological saline, regardless of CS exposure ($p < .001$). However, animals in the CTLS group showed trends toward lower values of torque removal when compared with those in the CTL group (Figure 5C).

Bone Metabolism Markers

In general, the nonstressed groups presented the highest values of BALP when compared with stressed animals. The AL group presented higher values when compared with ALS ($p < .001$) and CTLS ($p < .05$). The CTL group presented higher values in relation to CTLS ($p < .01$) and ALS ($p < .001$). No statistical differences were found between ALN and sterile physiological saline-treated animals not subjected to CS (Figure 6A).

Regarding CTX analysis, animals treated with ALN presented the lowest values when compared with control animals, independent of CS exposure ($p < .001$) (Figure 6B).

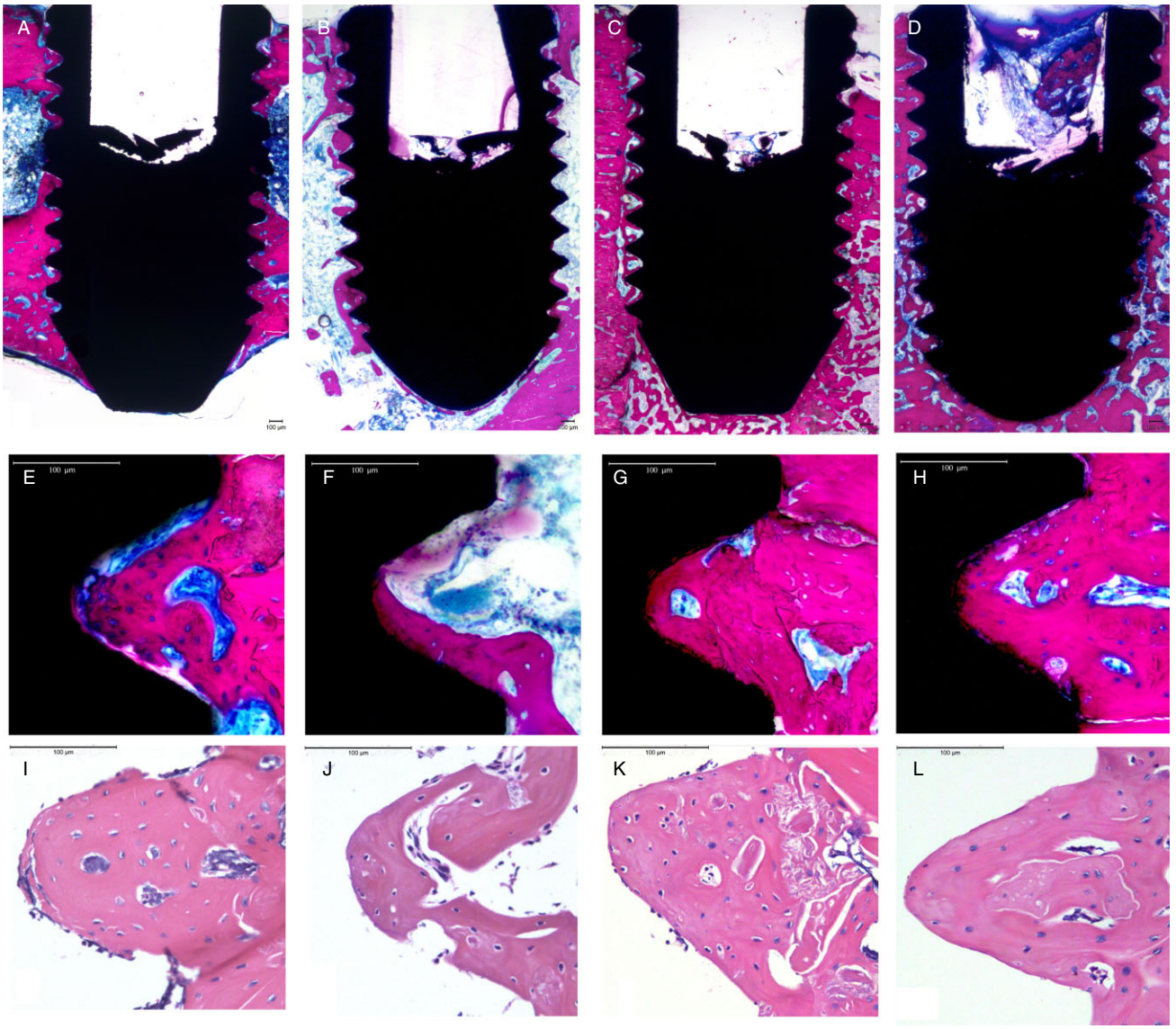

Figure 3 Histology aspects of implant cavity illustrated by longitudinal sections stained with hematoxylin and eosin (routine histology) and Stevenel's blue and acid fuchsin (hard tissue histology) 4 weeks after implantation. Animals of groups CTL (A, E, I) and CTLS (B, F, J) presented a high volume of spongious and marrow bone, which was observed in control animals (×25 and ×200). A high degree of compact bone was observed in groups AL (C, G, K) and ALS (D, H, L) (×25 and ×200). AL, alendronate; ALS, alendronate + stress; CTL, control; CTLS, control + stress.

Corticosterone Levels

Corticosterone levels were altered within weeks ($F[1.63] = 9.63$; $p < .001$), only in the animals exposed to CS. These values were significantly higher in the third and fourth weeks when compared with baseline and with values in the first stress cycle ($p < .05$). Furthermore, these rates were higher in relation to baseline and the first stress cycle of control animals ($p < .05$) (Figure 7).

DISCUSSION

In spite of the absence of studies investigating the relationship between stress and bone healing, the harmful effects of stress on soft tissue wound healing have been well established in the medical literature.[5–7] To the best of our knowledge, this is the first study to address and demonstrate the negative effects of CS on the osseointegration of titanium implants.

Corticosterone is the most abundant GC in rats,[23] and it has been considered a useful serum marker of the stress state in rodents.[19,24] Our findings of higher levels of plasma corticosterone in animals exposed to stressors confirm the efficiency of the stress paradigm. Furthermore, these outcomes also indicate that the animals did not adapt to the stress protocols during the

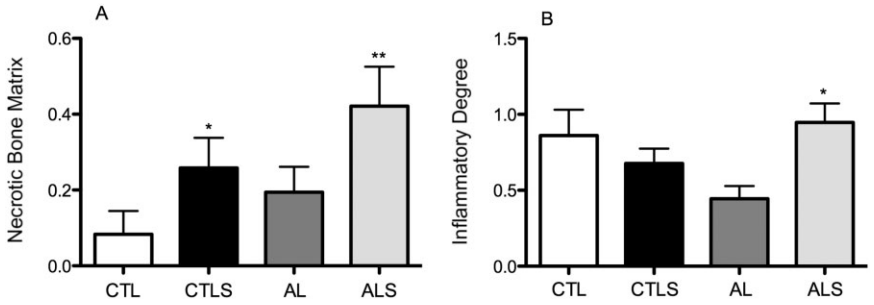

Figure 4 Histological features 28 days after tibial implant insertion. (A) Empty osteocytic lacunae proportion (*$p < .05$ and **$p < .01$ in relation to the CTL group). (B) Inflammation degree (*$p < .05$ in relation to the AL group). AL, alendronate; ALS, alendronate + stress; CTL, control; CTLS, control + stress.

osseointegration period, which is a relevant issue in CS models.

It is believed that the physiologic pathways of stress-induced wound-healing impairment could be related to enhanced GCs.[25,26] In this sense, in bone tissue, studies have shown that the administration of GC, especially long term, stimulates osteoclast-mediated bone resorption,[27] reduces osteoblast-mediated bone formation[28] and mineral bone density,[29] and is associated with the development of osteonecrosis.[30]

However, it is relevant to state that the effects of experimental stress should not be compared with those from the administration of exogenous steroids for two main reasons. First, stress is associated with changes in behavioral responses and other physiological processes, including catecholamine release, which are also related to wound-healing delay.[31] Second, the mean potency of synthetic steroids is significantly higher compared with that of endogenous steroids,[32] which means that a strong difference in the intensity of the physiologic effects of both hormone categories can be expected.

Indeed, in spite of histometric and biomechanical measures, only the BAFO values were significantly decreased in stressed animals with no ALN therapy. In our opinion, these effects can be related to an impairment of bone metabolism because the CS groups presented statistically lower values of BALP, even in ALN stressed animals. It is known that BALP is a product of

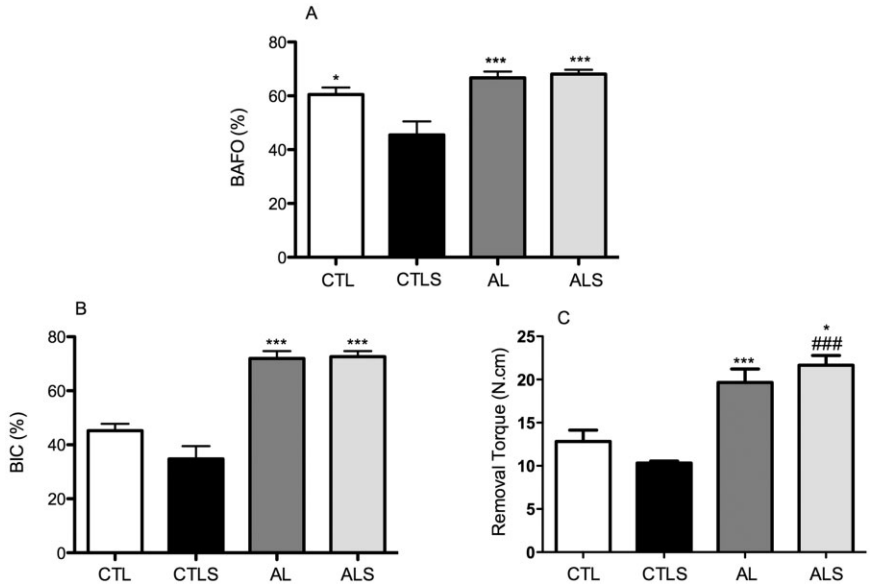

Figure 5 Histomorphometric measures 28 days after tibial implant insertion. Data shown as mean ± SD. (A) BAFO parameter (*$p < .05$ in relation to CTLS; ***$p < .001$ in relation to CTLS). (B) BIC parameter (***$p < .001$ in relation to CTL and CTLS). (C) Torque removal values (***$p < .001$ in relation to CTL and CTLS; ###$p < .001$ in relation to CTLS; *$p < .05$ in relation to CTL). AL, alendronate; ALS, alendronate + stress; BAFO, bone area fraction occupancy; BIC, bone-implant contact; CTL, control; CTLS, control + stress.

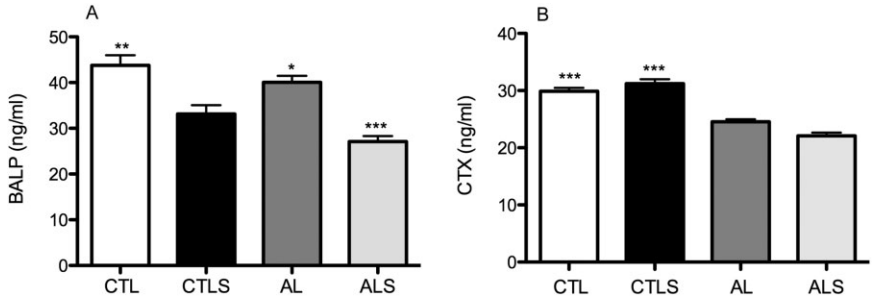

Figure 6 Concentrations of bone metabolism markers 28 days after tibial implant insertion. Data shown as mean ± SD. (A) BALP (***$p < .001$ in relation to CTL and CTLS; **$p < .01$ in relation to CTLS; *$p < .05$ in relation to CTLS). (B) CTX (***$p < .001$ in relation to AL and ALS). AL, alendronate; ALS, alendronate + stress; BALP, bone-specific alkaline phosphatase; CTL, control; CTLS, control + stress; CTX, C-telopeptide.

osteoblast activity and, therefore, a marker of bone formation.[33] In contrast, the CTX levels, which are products of osteoclast activity,[33] were not significantly different. Taken together, these findings suggest a partial impairment of bone healing around implants.

In contrast, in animals treated with ALN, none of the osseointegration parameters was different between nonstressed and stressed animals, even when considering that these latter animals had the lowest levels of BALP, as described previously. Furthermore, ALN therapy was associated with the highest values of histometric and biomechanical measurements. These findings are in agreement with those from other studies that confirm the potential of BPs to improve the osseointegration of implants,[13–15] including causing reversal of the bone loss induced by the administration of steroids to titanium implants[12] and by periodontal disease.[16]

However, the reasons for these positive effects have not yet been clearly established in the literature. While some authors justify these trends based on the suppression of the resorption process,[34] others showed that BPs stimulate new bone formation around implants.[35,36] Considering these studies and our findings, it is reasonable to believe that the inhibition of the resorption process may be a relevant factor because the lowest levels of CTX associated with similar levels of bone ALP were observed in ALN-treated animals, regardless of CS exposure.

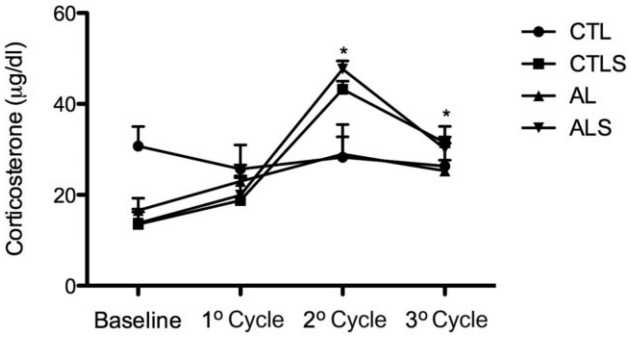

Figure 7 Plasma corticosterone levels in rats exposed to chronic stress during the experiment. Blood samples were collected at four time points: before stress exposition (baseline) and 24 hours after each cycle of stress. Data shown as mean ± standard error of the mean (*$p < .05$ in relation to baseline and 1° cycle of all groups). AL, alendronate; ALS, alendronate + stress; CTL, control; CTLS, control + stress.

Torque removal analysis is a test commonly performed to evaluate the strength of the bone-implant interface.[37–39] It has been demonstrated that ALN therapy significantly increases the values of implant torque removal.[14,40] This was also confirmed in our study, even in animals subjected to CS. In fact, the close relation between this biomechanical test and the degree of bone in contact with the implant[41,42] could support our findings of highest BIC values found in the ALN groups, as was also demonstrated by other authors.[14,40]

Nevertheless, it is relevant to state that bone mineral density (BMD) and bone architecture are key determinants of bone strength[43] and thus able to affect biomechanical implant tests. These observations can also explain the highest torque values in ALN-treated animals because we showed a high degree of compact bone close to the implants in these animals, which is also favored by the markedly increased BMD that is expected to occur during BP treatment.[44] In this way, associating these features with the higher BIC values found in ALN-treated animals, it could be speculated that BAFO measurement may not be a primary factor in torque removal analysis in the BP context because we found similar levels of BAFO between nonstressed control and AL groups.

Although our study clearly demonstrated positive effects of ALN on osseointegration, it is relevant to state that animals treated with these drugs presented the highest values of empty osteocytic lacunae areas, especially in the ALS group. Following the same tendency, the CTLS group also presented higher levels of empty osteocytic lacunae areas compared with the CTL group. The reasons that could explain these findings can be related to the potential pathways of both BPs and GC to induce osteonecrosis.[45–47] In fact, this association is particularly dangerous to the jaws because it has been shown to increase the severity of jaw osteonecrosis.[48]

Although necrotic cell death mediated by innate immune cells[49] can induce an inflammatory response, no differences were observed between CTL and CTLS groups. This is particularly interesting given the differences in the degree of empty osteocytic lacunae areas described above. One possible explanation for these findings could be related to the suppressive effects of CS on the immune response,[50] which might have an adverse effect on wound healing.[51]

On the other hand, no suppressive changes were observed in the degree of inflammation in the ALS group. It is likely that the higher inflammatory level observed in the ALS group would be related not only to the highest level of empty osteocytic lacunae areas but also to an additional effect of BP therapy in stimulating proinflammatory events.[52]

Several limitations remain to be acknowledged and addressed. First, the methodologies designed to address the osseointegration process do not elucidate the molecular pathways underlying the relationship between CS and implant wound healing, including the association with implant survival and long-term implant osseointegration. The second limitation is regarding to the extrapolation of outcomes from this animal study to humans. Animal models of stress are indispensable research tools to knowledge of mechanism involved in stress-induced pathologies. However, they have to be used with discernment and their predictive, face, and construct validities have to be examined before extending the data to humans.

CONCLUSIONS

Within the limitations outlined above, the present study showed, for the first time, the negative role of CS on the osseointegration of titanium implants in animals with no ALN treatment. Furthermore, the therapy with these drugs seems to improve osseointegration parameters and prevent the deleterious effects of stress. However, further studies, especially using jaw implants, are necessary to investigate the role of chronic BP therapy in implant survival and long-term implant osseointegration

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