

The Effect of Systemic Nicotine on Osseointegration of Titanium Implants in the Rat Femur

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The purpose of this study was to determine whether high levels of plasma nicotine, delivered via subcutaneously placed mini-osmotic pumps, had an effect on bone development and osseointegration of a titanium implant in rat femurs in both the short and long term. In this study, we hypothesized that systemic nicotine may not affect bone development, but may affect osseointegration in both the short and long term. Thirty rats were assigned to 4 groups. Group 1 (n = 10) was subdivided into 2 groups, which both received nicotine during the duration of the experiment. Half of the group (n = 5) was sacrificed at 2 weeks after implant placement, and the other half (n = 5) was sacrificed at 4 weeks after implant placement. Group 2 (n = 10) was treated identically; however, this group was given saline placebo rather than nicotine. Nicotine/saline was administered via subcutaneous mini-osmotic pumps. Serum analysis was assessed biweekly and weight was assessed weekly. Implant placement consisted of mini-implant placement in the femur of the rats under general anesthesia. After sacrifice, the femurs were harvested and analyzed. Biomechanical push-in test was used to determine the degree of osseointegration by evaluating the breakpoint load. Micro-CT was performed on the femurs of the remaining 10 rats to determine the bone density and architecture. Micro-CT showed no significant difference in bone morphometric analysis. Push-in test showed significant difference in axial load force required to dislodge the implant between the nicotine-treated and control rats both at 2 and at 4 weeks after implant placement. The evidence indicates that while there was no significant difference in bone development and remodeling with exposure to systemic nicotine, there was a significant difference in bone wound healing, specifically with the osseointegration of titanium implants at both 2 and 4 weeks after implant placement. In conclusion, systemic nicotine may have a significant impact on the osseointegration of implants in the rat femur. Additional studies need to be conducted to further understand the specific way in which nicotine adversely affects wound healing on the molecular level.

Key Words: *nicotine, dental implant complications, osseointegration, mini-osmotic pump, biomechanical push-in test, 3D CT, bone remodeling*

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INTRODUCTION

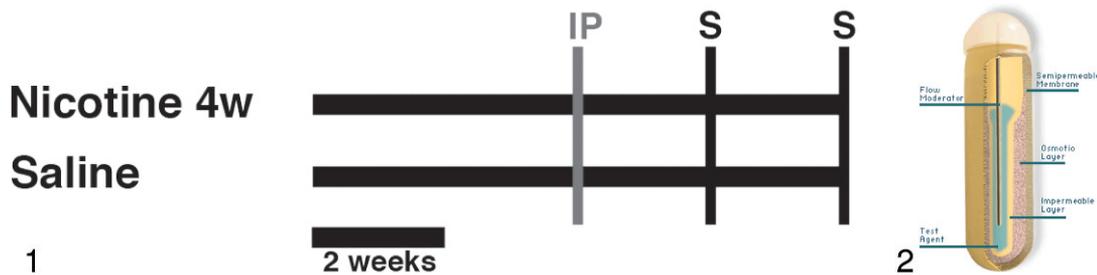
Endosseous titanium dental implants have been a primary tool for enabling tooth replacement for the past 30 years. They have been proven to be an overall safe and effective system for the replacement of unhealthy or missing teeth. The success of the endosseous implant relies primarily on bone wound healing and the ability of the alveolar bone to remodel and secure the titanium screw within the newly built bone. According to the vast majority of studies done looking at the variety of factors complicating implant success, smoking is considered a prominent risk factor.¹⁻³ Bain and Moy,¹ in the initial investigation into the effect of smoking on implants, looked at a group of 540 patients receiving a combined 2194 dental implants to better understand factors that predispose patients to implant failure. The most significant complication that they uncovered was that of smoking: a prevalent habit, which according to the American Heart Association in 2008, is performed by a significant portion of the American population (23.1% of men and 18.3% of women).

During cigarette smoking, there are nearly 4000 different gases that are released into the smoker. Being the first point of contact for these substances, the oral cavity is obviously susceptible to these destructive elements. These gases include nitrogen, carbon monoxide, carbon dioxide, ammonia, hydrogen cyanide, benzene, nicotine, nornicotine, anatabine, and anabasine.⁴ Tobacco's adverse effects on bone development and wound healing have been shown by tobacco being a significant risk factor for osteoporosis⁵ as well as being associated with reduced bone density in the femur, vertebrae, and jawbone.^{6,7} In addition, when comparing the bone mineral content between smokers and nonsmokers, it was seen that there was less bone mineral content in smokers.^{8,9} While

evidence has demonstrated the deleterious effects of smoking on health and healing, there is much that needs to be understood about the specific causative elements and biologic processes by which these effects are taking place.

The addictive element in tobacco, nicotine, has long been implicated in many disease processes and has been shown to be of the highest importance when understanding the negative effects of smoking on bones.¹⁰ While the specific mechanism of action that nicotine plays specifically on the activity of osteoblasts and osteoclasts is still unknown, there are elements of nicotine that do shed some light on its activity. For example, nicotine is known to reduce the proliferation of red blood cells, macrophages, and fibroblasts, which are important elements of healing.¹¹ In addition, nicotine increases platelet adhesiveness, which can lead to poor perfusion due to microclots.¹² Similarly, by stimulating the release of epinephrine and norepinephrine, nicotine acts as a sympathomimetic and causes increased vasoconstriction, which limits overall tissue perfusion.¹³ The connection between isolated nicotine and bone development can be illustrated in a study done by Boyne and Herford.¹⁴ The experiment involved stimulating subperiosteal bone development in rats. The study showed that in rats receiving nicotine via a plaster on their back, they had significantly less bone growth than those without nicotine, and even less bone growth than those simply exposed to cigarette smoke.

Many of the past studies that have isolated nicotine as their exposure agent and assessed its effect on osseointegration of titanium implants have found no significant difference in implant osseointegration between animals with or without exposure to nicotine.¹⁵⁻¹⁷ These studies have varied most significantly in their method of nicotine exposure as well as assessment of implant



FIGURES 1 AND 2. **FIGURE 1.** Outline of the experiment. IP indicates implant placement; S, sacrifice time. The bar represents 2 weeks. **FIGURE 2.** Alzet osmotic pump.

stability. The present study aims to assess the effects of systemic nicotine exposure, via subcutaneous mini-osmotic pump, on the osseointegration of implants in both the short and long term using the implant push-in test to assess biomechanical stability in the rat model, as set forth by Ogawa et al.¹⁸ This biomechanical stress test, which produces consistent load displacement measurement, is able to assess the degree of osseointegration by pinpointing the breakpoint at the implant tissue interface. The aim of the study was to shed light on whether nicotine was impacting osseointegration of implants in the short and long term using this biomechanical stress test, as well as using micro-CT to assess trabecular and cortical architecture to see the effects on bone development. We hypothesize that systemic nicotine exposure may not affect bone development, but that it may affect implant osseointegration in the rat femur.

MATERIALS AND METHODS

Animals and exposure

The experiment was performed on 30 male Sprague Dawley rats aged 4–6 weeks. The rats were divided into 2 groups; 1 group was exposed to nicotine while the other group was administered a saline placebo. Each group contained 2 subgroups of 5 rats: 1 subgroup that was sacrificed 2 weeks after implantation and 1 subgroup that was sacrificed 4 weeks after implantation (Figure 1). The remaining 10 rats were then

sacrificed at 8 weeks, and the femurs were harvested for micro-CT analysis. Each rat was weighed weekly, and the weights were plotted to compare those exposed to nicotine vs saline as well as to baseline values. Animal studies were performed under protocols approved by the Harvard Medical School Institutional Review Board.

Osmotic pump placement

The Alzet osmotic mini-pumps (Alzet model #2004, Alza Corp, Palo Alto, Calif) (Figure 2) were filled with either a nicotine solution or a nicotine-free/phosphate-buffered saline solution. Nicotine tartrate (Sigma Chemical Co, St Louis, Mo) was dissolved in sterile saline at a concentration to deliver an average of 6.0 mg nicotine/kg/d. After subcutaneous implantation, the pumps absorb extracellular fluid via osmosis, which then places pressure on the impermeable membrane that is surrounding the reservoir of nicotine. This method allows for a continuous dose of nicotine to be distributed to the animal. Prior to implant placement, the rats were anesthetized with an intramuscular injection of 100 mg/kg of ketamine and 10 mg/kg of xylazine. The osmotic pumps were surgically inserted subcutaneously between the scapulae. This model mini-pump delivers solution at a rate of 0.25 μ L/h for a period of up to 32 days. Although this continuous exposure to nicotine may seem to stray from the intermittent smoking patterns in humans, it has been shown that serum nicotine levels in smokers do remain elevated over a 24-hour period, and

thus a pulsed exposure was unnecessary.¹⁹ For both groups, the mini-pumps were replaced after the first 4 weeks, and a new one was inserted for the remaining weeks. This animal model has been well established and published extensively in the literature.^{15,16,20–23}

Serum nicotine and cotinine analysis

Nicotine was measured in blood samples at 4, 6, and 8 weeks. Serum levels of nicotine and cotinine were determined using gas chromatography (detection limit, 1 ng/mL) (Clinical Pharmacology Laboratories, University of California, San Francisco, Calif). Each 1-mL serum sample was processed for analysis of cotinine levels. Internal standard solution (ISTD) containing 5-methyl cotinine (or R,S-ortho-cotinine) was added to a 1-mL aliquot of sample submitted for testing. Each sample (and control) was mixed/vortexed and subjected to a multi-step partitioning process involving repeated liquid-liquid extractions and cryogenic freezing. The extracted sample was then analyzed by gas chromatography using split/splitless injection, a fused silica capillary column, and thermionic-specific detector. The analytes were quantified by comparing the unknown analyte/ISTD response ratio to a multi-point calibration curve of standards containing known concentrations of analyte.²⁴

Implant placement

General anesthesia was obtained via the aforementioned method of intramuscular injection of ketamine and xylazine. Skin was shaved and thoroughly cleansed with iodine surgical soap and 70% alcohol. The implants used were unthreaded cylindrical implants, 1 mm in diameter and 2 mm in length, with sandblasted acid-etched surface (given kindly by Straumann, USA). The implants were cleaned in 70% alcohol and sterilized via autoclave. A 1-cm incision was made across the dorsal surface of the both femurs (left

and right), and the surface of the bone was exposed with blunt dissection of the muscle and fascia layers. Monocortical implant sites were prepared under copious cooled saline irrigation at rotary speeds not exceeding 1500 rpm. The implants were placed in the osteotomy sites on the mesial surface of the femur with primary stability. Multilayer wound closure was performed by suturing muscle and skin tissue using 4-0 Vicryl and stainless steels wound clips, respectively. Following surgery, analgesics (buprenorphine, 0.05–0.1 mg/kg subcutaneously every 8–12 hours) were delivered to treat postoperative pain for the first 24–48 hours post operation. Sutures and clips were removed 7–14 days following the surgery.

Killing

At 2 and 4 weeks after implant placement surgery, rats were killed with overdose of carbon dioxide. The femurs containing implants were harvested and stored in phosphate-buffered saline at 4°C.

Micro-CT analysis

Desktop μ CT 40 system (Scanco Medical AG, Bassersdorf, Switzerland) was used for scanning as well as for morphometric analysis. All femur bones were placed in the holder and scanned transversely with the condyles in the inferior direction and the femur head in the superior direction. The scanned volume had the voxel resolution of 20 microns. These stacks of images were then passed through a 3D Gaussian filter. Segmentation was done by user-defined contouring followed by thresholding. Three-dimensional morphometric analysis was performed by choosing the trabecular region in the epiphyseal region and the cortical region and cavity in the diaphyseal region to get the bone volume fraction, trabecular thickness and structural modular index (obtained using global adaptive thresholding). The last phase involved a 3D visualization of the scanned samples (Figure 3).

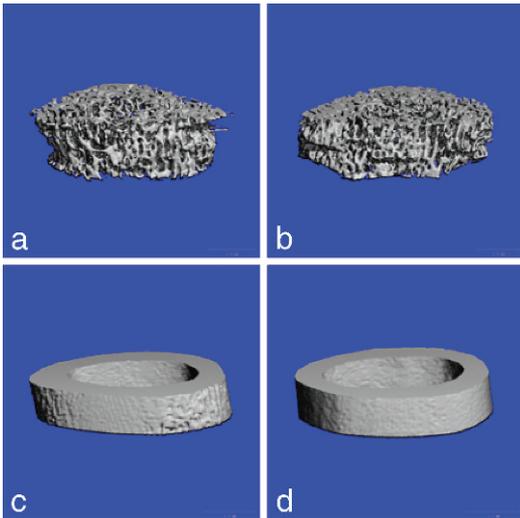
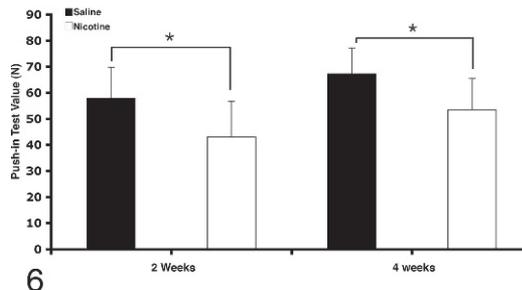
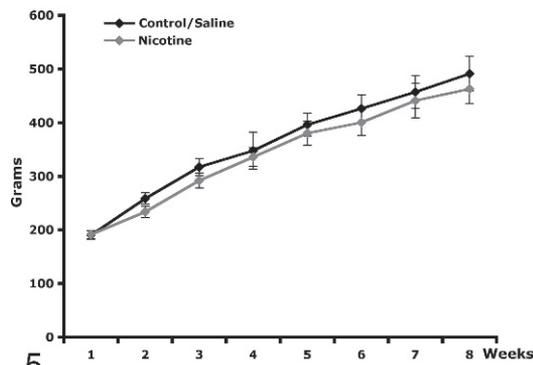
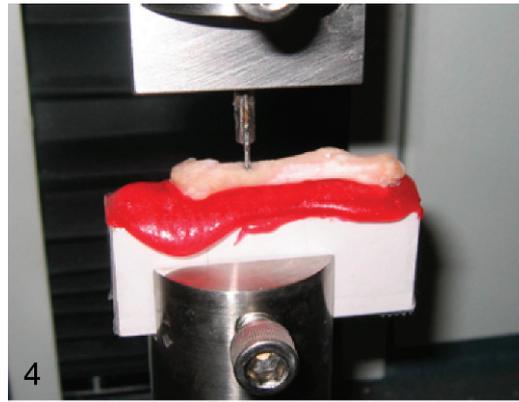


FIGURE 3. Three-dimensional structures of trabecular (a,b) and cortical bone (c,d) of the epiphyseal (a,b) and metaphyseal (c,d) areas between the control (a,c) and nicotine-treated rats (b,d).

Biomechanical stress analysis

After the femurs were harvested and all soft tissue was removed, the femurs were transported in sterile saline and immediately embedded in autopolymerizing resin (GC pattern resin, GC Corporation, Tokyo, Japan) in a custom-made mold that allowed for the femurs to be perpendicular to the load applied by a testing machine (Instron 5544 Electro-Mechanical Testing System, Instron, Canton, Mass) and a direct axial load to the implants (Figure 4). Prior to the push-in test, the femurs were viewed by radiograph to examine the position and inclination of the implants. The testing machine consists of a stainless steel pushing rod with a diameter of 0.8 mm. For each sample, the femur and mold were placed in an adjustable mount and centered beneath the pushing rod. An axial load was applied to the implant at a rate of 1 mm/min. The push-in value was determined as the fracture point, or the maximum load applied before a rapid decrease in load force was observed, signifying a break in the implant-bone contact surface. In this way, a load displacement curve with specific breakpoint values was generated for each implant tested. This force



FIGURES 4–6. **FIGURE 4.** Biomechanical test (push-in test) will be performed to measure the osseointegration level. **FIGURE 5.** Weight measurement over 8 weeks in the nicotine and control groups. **FIGURE 6.** Implant push-in test. * $P < .05$ ($n = 10$ implants/group).

was able to assess mechanical strength and thereby determine levels of osseointegration.

Statistical methods

The data that were collected over the course of the study were entered into an electronic database created using a commercially available statistical software program (SPSS v. 11.0, SPSS Inc, Chicago, Ill). Statistical mean, range, and standard deviation were computed for the study groups. Bivariate analysis was

| | Control (Saline) | Nicotine | | |
|------------------|------------------|---------------|--------------|--------------|
| | | 4 Weeks | 6 Weeks | 8 Weeks |
| Nicotine (ng/mL) | 0 | 93 \pm 12 | 126 \pm 45 | 105 \pm 27 |
| Cotinine (ng/mL) | 0 | 327 \pm 102 | 318 \pm 51 | 261 \pm 37 |

conducted to determine statistical equivalence between study groups. The Tukey least significant difference procedure was used to identify specific differences between groups. For all analyses, P values $<$.05 were considered statistically significant.

RESULTS

Serum analysis

Serum analysis was conducted to assess that the rats were receiving the proper amount of nicotine that would be expected over the short and long term. The serum nicotine and cotinine levels (mean ng/mL \pm SD) showed 92 \pm 12 and 327 \pm 102 at 4 weeks, 126 \pm 45 and 318 \pm 51 at 6 weeks, and 105 \pm 27 and 216 \pm 37 at 8 weeks, respectively. Samples from the control rats were also sent for analysis and were confirmed to contain no nicotine or cotinine (Table 1).

Weight analysis

The graph in Figure 5 consists of the body weights over the 8-week experiment of the rats for both the nicotine and the saline group. Black dots represent mean in grams \pm SD of control groups, and gray dots

represent mean in grams \pm SD of nicotine delivered. The graph demonstrates a lower weight in the group that was administered nicotine as compared with the group administered saline; however, the difference was not statistically significant (Figure 5).

Micro-CT analysis

Three-dimensional visualization using micro-CT showed no difference detected in trabecular and cortical bone architecture between control and nicotine-exposed groups (Figure 3). In 3D morphometric analysis, there is no significant difference regarding bone volume and trabecular number for both the mid shaft and epiphyseal region between the control and nicotine-exposed groups (Table 2). The mean morphometric values (mean \pm SD) in the mid shaft region for the control and nicotine-exposed groups were 0.523 \pm 0.040 and 0.508 \pm 0.030 in bone volume (%), respectively; 2.374 \pm 0.198 and 2.419 \pm 0.072 in trabecular number (mm), respectively; and 0.549 \pm 0.040 and 0.527 \pm 0.029 in trabecular thickness (mm), respectively. The mean morphometric values in the epiphyseal region for the control and nicotine-exposed groups were 0.257 \pm 0.019 and

| Endpoint | Control (Saline) | Nicotine |
|---------------------------|------------------|-----------------|
| Mid shaft region | | |
| Bone volume (%) | 0.52 \pm 0.04 | 0.50 \pm 0.03 |
| Trabecular number (mm) | 2.37 \pm 0.19 | 2.41 \pm 0.07 |
| Trabecular thickness (mm) | 0.54 \pm 0.04 | 0.52 \pm 0.02 |
| Epiphyseal region | | |
| Bone volume (%) | 0.25 \pm 0.01 | 0.24 \pm 0.03 |
| Trabecular number (mm) | 4.69 \pm 0.19 | 4.19 \pm 0.77 |
| Trabecular thickness (mm) | 0.07 \pm 0.00 | 0.07 \pm 0.00 |

0.244 ± 0.038 in bone volume (%), respectively; 4.69 ± 0.199 and 4.19 ± 0.771 in trabecular number (mm), respectively; and 0.077 ± 0.002 and 0.075 ± 0.002 in trabecular thickness (mm), respectively. There is no significant difference regarding bone volume and trabecular number and thickness in both the mid shaft and epiphyseal region between the 2 groups in 3D morphometric analysis.

Implant push-in test

The biomechanical stress test to assess the implant stability at 2 and 4 weeks after implant placement shows a significant difference in axial load required to dislodge the implant. In biomechanical stress analysis, the mean breakpoint values (mean N \pm SD) for the control and nicotine-exposed groups were 57.8 ± 12.0 and 43.2 ± 13.5 at 2 weeks, respectively, and 67.3 ± 9.9 and 53.6 ± 12.0 at 4 weeks, respectively (Figure 6). The mean of the nicotine-group yielded significantly lower push-in values at both 2 and 4 weeks than the control group ($P < .05$). This indicates a lower rate of osseointegration in the nicotine-exposed rats than in the rats administered the saline placebo.

DISCUSSION

These results indicate that although bone development seems to be unaffected by systemic nicotine in the rat model, it is evident that nicotine does in fact play a significant role in wound healing, specifically in the osseointegration of titanium implants. While there was no significant difference in the weights between the nicotine and saline groups, there was a slight discrepancy that indicated that nicotine did cause some systemic effects leading to relatively lower body weight in those animals. In one previous study using nicotine of various proportions (4.5 or 6 mg/kg/d), it was seen that in rabbits exposed to nicotine for

3 months, there was 7% less body weight than the control group.¹⁹ In another study with rabbits, administering nicotine for 4–6 weeks (8.6 mg/kg/d) also showed a significant effect on body weight when compared with controls. Since one study showed longer duration while another showed greater dosage, the impact of nicotine on body weight may be related to duration and dosage of nicotine exposure.

The serum analysis shows that a baseline level of nicotine was indeed maintained throughout the exposure period and that the control rats had no nicotine or cotinine in their serum at any time. This systemic nicotine exposed the test sample to various effects not seen in the control sample. In terms of the bone development and remodeling, as assessed by analyzing the femurs with 3D computed tomography, no negative effects were seen in the sample exposed to nicotine as compared with the control sample. There was no effect on bone volume percent, trabecular number, or trabecular thickness on 3D morphometric analysis. These findings confirm those of prior studies such as one that investigated the effects of nicotine inhalation on bone mass and mechanical properties in the femurs of female rats.²⁵ Another study which supports this demonstrated that while estrogen-depleted rats showed significantly less bone mass and strength, rats exposed to nicotine via mini-osmotic pumps displayed no significant difference with the control. It can be concluded that as long as rats are healthy, excluding factors such as estrogen-depletion and old age, nicotine administration in a short term may not affect bone development and remodeling.

While the effects of nicotine were not seen with bone remodeling, they were seen when investigating bone wound healing. As shown in the biomechanical push-in test, the axial forces required to dislodge the implants were significantly higher in the control sample than

in those exposed to nicotine. It can be seen from this that at 2 and 4 weeks, the osseointegration was indeed more complete, and thus implant stability more reliable, when exposed to saline versus nicotine. Previous similar studies have not come to this same conclusion. One study demonstrated no significant impact of short exposure to high doses (8.6 mg/kg/d) of nicotine in the rabbit on the osseointegration of titanium implants in the femur and tibia.¹⁶ Their analysis of implant stability consisted of resonance frequency analysis, removal torque test, bone area within the threads, and percent bone: implant contact. Potentially, the species difference or method of analysis may be related to the discrepancy in the determination of the impact of nicotine on osseointegration.

Looking at humans, it has been demonstrated that smoking was a significant factor in the failure of implants prior to functional loading.²⁶ Nicotine's clear role in this process is still incompletely understood, and further investigation is required. In trying to understand the physiology behind nicotine's molecular effects, one *in vitro* study attempts to shed light on this issue by investigating the overall effect of nicotine on human bone marrow cells that were cultured around titanium implants. They concluded that the high tissue diffusion potential of nicotine suggests the possibility of a direct modulation of osteoblast activity as a contributing factor to nicotine's effect on the bone microenvironment around implants.²⁷

CONCLUSION

This study shows the detrimental impact that nicotine and smoking can have on implant osseointegration, stability, and success. While it remains difficult to draw direct parallels between an animal model and clinical correlations, extensive literature has been published on the efficacy and necessity

of smoking cessation protocols prior to implant placement. Most notably, in an investigation into smoking cessation protocols, Bain²⁸ demonstrated that in the group that underwent a cessation period of 1 week prior and 8 weeks after implant placement, there was significantly less implant failure than in the group of smokers who continued smoking. It seems to this author, that given the data seen in the animal model, smoking, and specifically any form of ingestion of nicotine, should be considered a clear risk factor for implant failure. Therefore, patients should be informed and counseled so that they understand the risks and can modify their behavior accordingly. Further studies are needed to expound upon this research in order to fully understand the role that nicotine is playing in this biological process of bone wound healing. Ground sections to assess bone:implant contact ratios as well as SEM analysis of the implant surfaces would give a greater understanding of the patterns and processes of osseointegration. In addition, bone matrix-related gene expression could help us better understand the factors within the bone that are being regulated by nicotine. Due to the prevalence of nicotine exposure within this country and the international community at large, it remains increasingly vital for the scientific community to further the understanding of the effects and risks that this behavior imposes in order to better counsel and treat these individuals. In summary, these findings illustrate the negative effect that nicotine plays on wound healing and osseointegration of titanium implants using a rat femur model. Further studies of the detailed mechanisms at the molecular levels are necessary to fully understand how nicotine is impacting this process.

ABBREVIATION

ISTD: internal standard solution

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