



RESEARCH ARTICLE

Strontium-Loaded Titanium and Systemic Teriparatide Synergistically Enhance Osteogenesis and Osseointegration in an Osteoporotic Rabbit Model

Koichiro Shima¹ | Takayoshi Shimizu¹ | Seiji Yamaguchi² | Bungo Otsuki¹ | Toshiyuki Kawai¹ | Yaichiro Okuzu¹ | Yusuke Takaoka¹ | Norimasa Ikeda¹ | Shuichi Matsuda¹

¹Department of Orthopaedic Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan | ²Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai, Aichi, Japan

Correspondence: Takayoshi Shimizu (takayosh@kuhp.kyoto-u.ac.jp)

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ABSTRACT

Poor osseointegration of Titanium (Ti) implants in osteoporotic bone can lead to early construct failure in clinical settings. This study investigated whether combining Strontium (Sr) surface loading (modified alkali and heat treatment) with systemic teriparatide administration could enhance implant osseointegration in osteoporotic conditions. Mouse osteoblast-like cells (MC3T3-E1) were cultured on Sr-loaded Ti surfaces with and without teriparatide administration to evaluate cell adhesion, proliferation, differentiation, and mineralization capacity. This in vivo study utilized an osteoporotic rabbit femur through ovariectomy and steroid administration. The combined treatment (Sr-loaded Ti and teriparatide) enhanced osteoblast differentiation and mineralization in vitro, with an increase in alkaline phosphatase activity and alizarin red staining. Six and twelve weeks after in vivo implantation, the combination therapy demonstrated superior outcomes compared to the single treatments (Sr-loaded Ti or teriparatide), including enhanced bone–implant interfacial strength, improved bone morphology parameters, higher mineral apposition rates, and greater bone–implant contact. These findings demonstrate that the synergistic approach of Sr-loaded Ti implants combined with systemic teriparatide administration considerably improves implant osseointegration in osteoporotic bone, suggesting a promising strategy for enhancing implant outcomes in patients with osteoporotic bone quality.

1 | Introduction

Osteoporosis is a highly prevalent metabolic bone disease and is characterized by reduced bone mass and density. This condition leads to fragile bones that are vulnerable to fractures [1]. With the aging population, the global prevalence of osteoporosis is reported to be 19.7% [2]. The insertion of implants into osteoporotic bone during orthopedic and dental surgeries poses a significant challenge due to the increased risk of early migration or loosening [3–5]. Therefore, implants with enhanced early

osseointegration capabilities are essential, particularly for osteoporotic settings.

Commercially pure titanium (Ti) and Ti alloys are commonly employed in fabricating metallic implants due to their favorable biocompatibility, corrosion resistance, and mechanical strength [6]. Prior studies have investigated enhanced osseointegration using alkali and heat treatment (soaking in a NaOH solution at 60°C for 24 h and then heating to 600°C) on pure Ti and Ti alloys [7–9]. This process forms a bone-like apatite layer on the surface

at the submicrometer scale, enabling direct bonding between the Ti and living bone [8, 9].

Strontium (Sr) has been used in clinical practice as an anti-osteoporotic agent in the form of Sr ranelate to prevent vertebral fractures in post-menopausal women [10]. Sr can promote bone formation by activating the calcium-sensing receptor and MAPK ERK1/2 and Wnt/NFATc signaling to enhance osteoblast differentiation. Sr also inhibits bone resorption by reducing the receptor activator of the nuclear factor- κ B ligand (RANKL) expression and increases osteoprotegerin expression by osteoblasts/stromal cells; this antagonizes soluble RANKL, resulting in reduced pre-osteoclast differentiation into osteoclasts [11, 12]. Our previous studies using Sr-loaded Ti implants (a modification of the abovementioned alkali and heat treatment) have demonstrated that localized gradual Sr release can further promote in vitro osteogenesis and in vivo osseointegration [13]. This Sr treatment can be applied to grade 5 Ti alloys (90% Ti, 6% Al, and 4% V (Ti6Al4V)) metals and three-dimensional (3D) printed porous Ti using selective laser melting [14, 15].

Further, teriparatide (TPTD), a recombinant human parathyroid hormone consisting of the 1–34N-terminal fragment, has been demonstrated to increase osteoblast number and stimulate bone formation through multiple mechanisms, including canonical Wnt signaling potentiation and RANKL reduction [16]. Intermittent TPTD administration increases vertebral and femoral bone mineral density, resulting in risk reduction for vertebral and other fractures [17]. Furthermore, studies have shown that intermittent TPTD administration may improve implant osseointegration in osteoporotic animal models [18–27].

We hypothesized that combinatorial treatment with Sr-loaded Ti implants and intermittent TPTD injections may exert synergistic effects to improve bone-implant osseointegration as well as bone quality surrounding implants. This study evaluated the efficacy of this dual regimen in enhancing osseointegration in an ovariectomized rabbit model of postmenopausal osteoporosis and investigated possible cooperative interactions on osteogenesis underlying mechanisms using in vitro assays.

2 | Materials and Methods

2.1 | Surface Treatment and Characterization

2.1.1 | Sample Preparation

Commercially pure Ti (ISO5832-2) (Ti; >99.5%; Nilaco Co., Tokyo, Japan) was cut into disc shapes (18×1 mm, d×h) for the in vitro tests and surface-characterization analyses and into cylindrical shapes (6×15 mm, d×h) for the in vivo tests (Fig. 1a). Ti and Sr-loaded Ti (Sr-Ti) were used in the experiments. For Sr-loading treatment, Ti was initially treated with 5 M NaOH (Kanto Chemical, Tokyo, Japan) at 60°C for 24 h, and then soaked in 50 mM CaCl₂ (Kanto Chemical) + 50 mM SrCl₂ (Kanto Chemical) at 40°C for 24 h, exposed to 600°C for 1 h, and treated with 1 M SrCl₂ [28]. All substrates were carefully cleaned with acetone, 2-propanol, and ultrapure water for 30 s in an ultrasonic cleaner and then dried. Before in vitro and in vivo experimentation, both samples were sterilized using ethylene oxide gas.

2.1.2 | Surface Characterization

The surface morphology was analyzed using a field emission scanning electron microscope (FE-SEM, S-4300, Hitachi Co., Tokyo, Japan) to study the changes in surface morphology induced by chemical solution and heat treatment.

The chemical surface elements of the metal samples subjected to chemical and heat treatments were analyzed by an energy-dispersive X-ray spectrometer (EDX; EMAX-7000, Horiba Ltd., Kyoto, Japan) at 9 kV for Ca, Sr, O, and Ti in five areas, and the average value was used for the analysis.

2.1.3 | Sr-Ion Release

The disc samples that were subjected to chemical and heat treatments were next soaked in 4 mL of phosphate-buffered saline (PBS) at a specific concentration (Na⁺ 158.14, K⁺ 1.06, Cl[−] 155.17, and HPO₄^{2−} 4.03 mM), and the pH was adjusted to 7.4 at 36.5°C. The samples were then gently shaken at 50 strokes per minute for various durations of up to 14 days. At predetermined time intervals, the PBS was refreshed. After soaking in PBS, inductively coupled plasma-emission spectroscopy (ICP; SPS3100, Seiko Instruments Inc., Japan) was used to measure Sr-ion concentrations in PBS. Three independently prepared samples were measured, and the released Sr concentrations were calculated by subtracting background values (taken by measuring PBS shaken under the same conditions without immersing the samples).

2.2 | In Vitro Study

2.2.1 | Cell Culture

All in vitro tests were conducted using a mouse calvaria-derived osteoblast-like cell line, MC3T3-E1. The cells were incubated in α -minimal essential medium (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (FBS) (lot number S0891; MP Biomedicals, Irvine, CA, USA) at 37°C in 5% CO₂ until they reached 80% confluence. At that point, they were used for subsequent experiments.

In 200 μ L of culture medium, 1×10⁴ cells were seeded on the disc-shaped implants in 12-well plates. Following a 3 h incubation period, 800 μ L of culture medium was added, resulting in a total volume of 1 mL for the cells. For the intermittent TPTD treatment group, the cells were incubated with 10 nM TPTD (teriparatide acetate; Asahikasei Pharma Corporation, Tokyo, Japan) for 6 h throughout each 48 h incubation cycle [29, 30]. In the control group, the culture medium was replaced every 48 h, starting simultaneously with TPTD media exchange. Ti and Sr-Ti with and without TPTD treatment were compared (Ti, Sr-TPTD-Ti, and TPTD-Sr groups, respectively).

2.2.2 | Cell Adhesion Using a Scanning Electron Microscope (SEM)

Cells were seeded and cultured as described in 2.2.1. Following a 24 h incubation period, each sample was subjected to a 2 h fixation

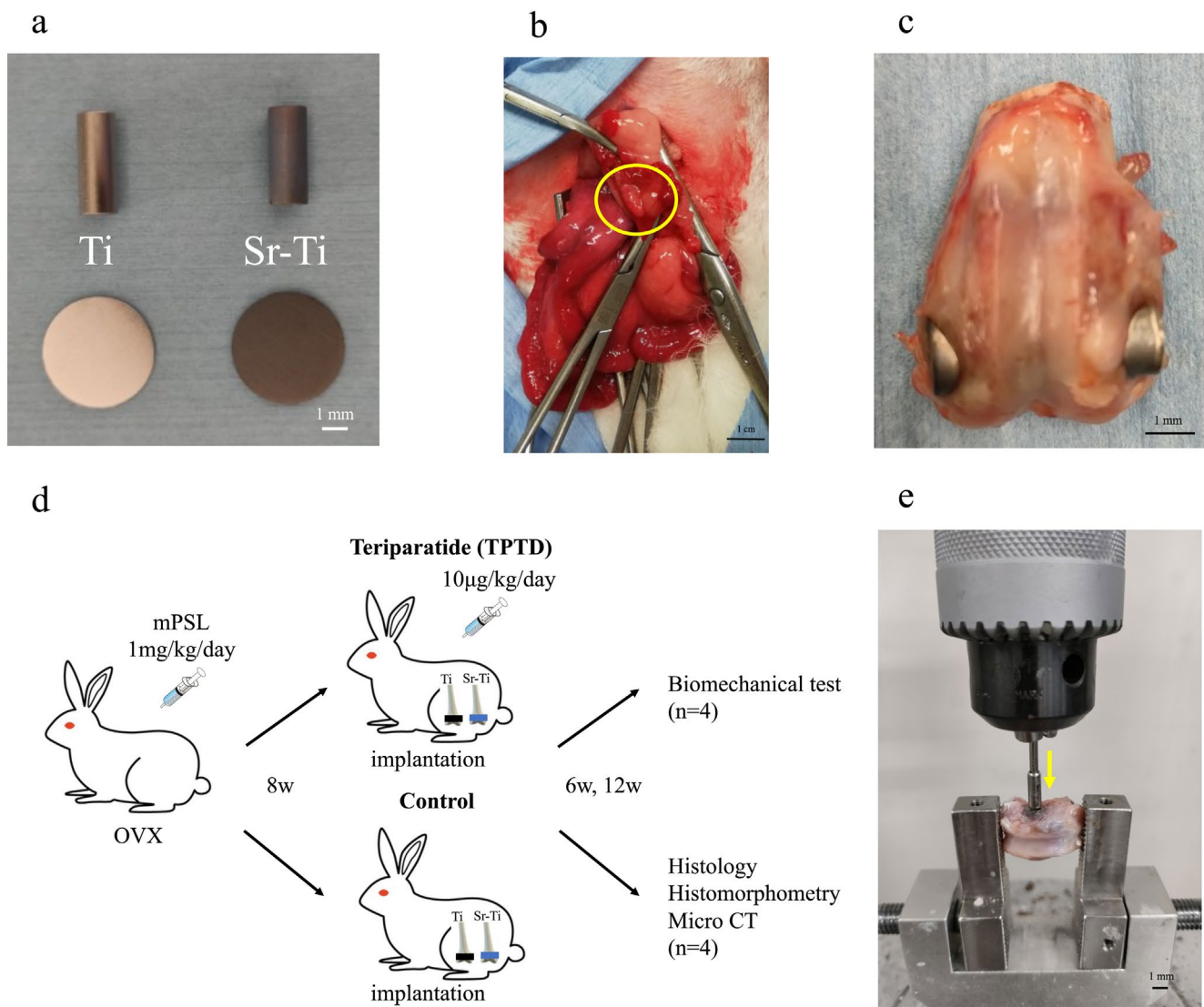


FIGURE 1 | (a) Ti (untreated) and Sr-loaded Ti (Sr-Ti) implants used in this study; cylindrical implants (6 × 15 mm, d × h) for in vivo tests, and disc-shaped implants (18 × 1 mm, d × h) for in vitro tests. Scale bar: 1 mm. (b) Intraoperative photograph of a rabbit ovariectomy (OVX). The yellow circle is the left ovary. Scale bar: 1 mm. (c) The model for the in vivo test. A cylindrical implant is inserted into the distal femur of a rabbit. Scale bar: 1 cm. (d) Flowchart of the experiments. (e) Push-out test method. The specimen is fixed, and a 5 mm rod parallel to the implant is pushed in the direction of the yellow arrow from above. Scale bar: 1 mm.

process at 4°C on ice, using 2.5% glutaraldehyde. Subsequently, the samples were dehydrated with progressively elevated ethanol concentrations for 10 min each, then dried with hexamethyldisilazane. All samples were coated with a thin Pt-Pd film and examined under an SEM (S-4700; Hitachi Ltd., Tokyo, Japan).

2.2.3 | Fluorescent Immunostaining of the Actin Filament

As described in 2.2.1, cells were seeded and cultured. Following a 24 h incubation period, the cells were fixed in 4% paraformaldehyde for 10 min, after which they were incubated in 0.1% Triton X-100 for a further 10 min. A blocking step with 4% FBS followed for 30 min, and the samples were then incubated with rhodamine-phalloidin (Cytoskeleton, Denver, CO, USA) for 20 min to label the cell cytoskeleton and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA,

USA) for 10 min to identify the nucleus. The stained cells were examined under a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan).

Five cells were randomly selected from each 10× image to quantify cell shape, and the length/width aspect ratio was calculated using a two-dimensional (2D) image processing software (ImageJ; National Institutes of Health, Bethesda, USA) [31]. To count the number of adherent cells on the substrate, five 10× DAPI-stained images were randomly selected on each implant surface, and nuclei were counted using Image J software [15].

2.2.4 | Cell Proliferation

Cells were seeded and cultured as described in 2.2.1. After one and seven days of culture, the XTT working solution (Roche

Diagnostics GmbH, Mannheim, Germany) was added to all substrates, which were then incubated for 3 h at 37°C. Subsequently, the absorbance of the transferred samples (150 µL) was measured at both 405 and 620 nm on a 96-well plate using a microplate reader (iMark Microplate Absorbance Reader; Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.2.5 | Alkaline Phosphatase Activity (ALP) Assay

As detailed in Section 2.2.1, the cells were subsequently seeded and cultured. LabAssay ALP (Wako Pure Chemical Industries, Osaka, Japan) was utilized in this investigation. Following a 30 min incubation period at 37°C with 6.7 mM p-nitrophenyl phosphate (pNPP), the pNPP at 405 nm was determined via a microplate reader (iMark Microplate Absorbance Reader), and ALP activity was subsequently calculated using a standard curve. ALP levels were normalized to total protein and compared to a control (Ti) group.

2.2.6 | Alizarin Red Staining

Cells were seeded as described in 2.2.1. An osteogenic medium containing 0.2% β-glycerol phosphate, 0.2% hydrocortisone, and 1% ascorbic acid (Takara-bio Inc., Shiga, Japan) is used in this experiment. This investigation utilized the Alizarin Red S staining kit (Bio Future Technology, Tokyo, Japan). Briefly, after a 21 d culture, the cells were washed twice with PBS, fixed in propylene glycol for 30 min, and washed twice with distilled water. The cells were stained with alizarin red for 30 min and washed twice with distilled water. For quantitation, the stained calcium nodules were dissolved in 5% formic acid, and the optical density at 490/650 nm was subsequently measured (iMark Microplate Absorbance Reader). The values were expressed in relation to that of Ti.

2.3 | In Vivo Study

2.3.1 | Experimental Animals

The experimental procedure was approved by the Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan (approval number: MedKyo23566). Thirty-eight 17-week-old female Japanese white rabbits (weight 2.6–3.4 kg) were used. The rabbits were housed in a standard rabbit cage and provided with standard food and water ad libitum.

2.3.2 | Rabbit Osteoporosis Model

To induce osteoporosis, the rabbits were subjected to bilateral ovariectomy (OVX). The rabbits were anesthetized with an intravenous administration of 40 mg/kg sodium pentobarbital (Ravonal; Nipro, Osaka, Japan); maintenance anesthesia was provided by isoflurane (Viatris, Tokyo, Japan) inhalation and local anesthesia with 1% lidocaine (Viatris, Tokyo, Japan). The rabbit was placed in a supine position, and a 3 cm vertical skin incision was made in the midline of the abdomen. A longitudinal

incision was made along the white line of the rectus abdominis muscle. The ovarian tubes were intraperitoneally maneuvered out of the uterus to expose the bilateral ovaries. The ovarian arterioles were hemostatic with a bipolar, and the ovaries were dissected. The rectus abdominis muscle and skin were sutured with 4–0 Ethilon (Johnson and Johnson, New Brunswick, NJ, USA) (Figure 1b). Two weeks after OVX, the rabbits received daily intramuscular injections of 1 mg/kg of methylprednisolone (Solu-Medrol; Pfizer, New York, NY, USA) for four consecutive weeks [19, 32, 33].

To confirm the osteoporotic model, six rabbits were randomly assigned to two groups and subjected to either the OVX + PSL (osteoporosis) or sham (with saline injection) treatment 8 weeks after OVX. A micro-CT scanner (SkyScan 1275, Bruker, Kontich, Belgium) was employed to assess bone mineral density (BMD). All specimens underwent scanning at a pixel resolution of 15 µm, with image acquisition at 80 kV and 80 µA. Following scanning, all images were reconstructed using the NRecon software (ver. 1.7.4.2, Bruker). Two phantoms with known BMD values (0.25 and 0.75 g/cm³) were imaged under the same conditions for calibration against their attenuation coefficients. The CTAn software (ver. 1.17.7.2, Bruker) was used to evaluate BMD for cortical and trabecular bone together at 3 mm proximal to the distal femoral epiphyseal line.

2.3.3 | Surgical Procedure of Implantation

Eight weeks after OVX, the implants were inserted into the distal femur of all rabbits. Rabbits were anesthetized as described in section 2.3.2 and were placed in a supine position. A straight incision measuring 3 cm was made over the lateral femoral condyle. The fascia was incised, and the attachment of the lateral collateral ligament was stripped. An electric bar with a 1 mm diameter was used to create a downhole from the lateral condyle to the medial condyle. The hole was then widened sequentially to 6 mm with a hand tap. After irrigating the hole with saline, a cylindrical material (6 × 15 mm, d × h) was implanted into the hole. The fascia and the skin were closed with 4–0 Ethilon sutures. The surgical procedures were performed bilaterally. In a randomized manner, one femur was implanted with a Ti implant, while the other femur was implanted with a Sr-Ti implant (Figure 1c) [34, 35].

2.3.4 | Treatment After Implantation

Thirty-two rabbits were randomly assigned to two groups: the control group was not subjected to any intervention, while the TPTD group received intermittent subcutaneous injections of TPTD at a dosage of 10 µg/kg/d, administered 5 d per week up to the day before euthanasia [19, 36]. A biomechanical, radiological, and histological analysis was conducted on Ti, Sr, TPTD-Ti, and TPTD-Sr groups at 6 and 12 weeks post-implantation. Four rabbits were subjected to biomechanical evaluation at each designated time point, while four additional rabbits underwent radiological and histological analysis concurrently. For histomorphometry, subcutaneous fluorochrome alizarin red (Sigma-Aldrich) was administered at a dose of 30 mg/kg to each animal 14 d before euthanasia.

Additionally, calcein (Matrix Scientific, Columbia, SC, USA) at a dose of 20 mg/kg was administered to each animal 7 d before euthanasia. The flow chart of the experiment is shown in Figure 1d.

2.3.5 | Biomechanical Testing

Push-out tests were conducted within 24 h of euthanasia at room temperature to ascertain the strength of the bone–implant interface attachment [37]. An Instron-type autograph (Model 1011; Aikoh Engineering, Aichi, Japan) was used. The orientation of each specimen was meticulously adjusted to ensure that the push-out direction was parallel to the contact surface between the implant and the bone. The bone sample was secured in the bilateral holder, and the cylindrical implants were extracted from the bone sample by a 5-mm-diameter rod gripped in a drill chuck with a crosshead speed of 1 mm/min. The maximum force was measured (Figure 1e).

2.3.6 | Radiological Analysis

The specimens were scanned using a micro-CT scanner (SkyScan 1275, Bruker) at a pixel resolution of 15 μm with image acquisition at 80 kV and 80 μA through a 1 mm copper filter, incorporating a rotation step of 0.2 mm and a frame averaging of 4 in 360 scan mode. After scanning, all images were reconstructed using the NRecon software (ver. 1.7.4.2, Bruker). The reconstruction was performed with a beam-hardening correction of 25%, a smoothing of 1, and a ring-artifact correction of 6. The Data Viewer software (Version 1.5.6.2, SkyScan) reconstructed the images in three planes (transverse, longitudinal, and sagittal) relative to the implant. The CTAn software (ver. 1.17.7.2, Bruker) was used to evaluate the bone around the implant. Regions of interest (ROI) were determined in an axial view of the implant using the custom processing tool. The initial step involved applying a binary threshold using a grayscale value between 250 and 255, thereby selectively identifying the metallic implant, followed by the removal of any residual pores and a three-pixel expansion. The image was designated as “ROI internal” and underwent another dilation process, this time by 7 pixels. This new threshold was designated as “ROI external”. The final ROI was set by subtracting “ROI internal” from “ROI external”. A new threshold was set to evaluate and isolate the bone, utilizing grayscale values of 100–255. This final volume of interest (VOI) was then subjected to a 3D analysis of the peri-implant bone, measuring bone volume/tissue volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, 1/mm), and trabecular separation (Tb.Sp, μm) [18, 21].

2.3.7 | Histology and Histomorphometry

After micro-CT analysis, the specimens with an implant were fixed in phosphate-buffered 10% formalin (pH 7.4) for 10 d, after which they were dehydrated in ascending concentrations of ethanol. They were then defatted in ascending concentrations of styrene and embedded in polyester resin. The specimens were bisected into slices of approximately 900 μm in thickness using a band saw (BS-3000 CP; EXACT Cutting Systems, Norderstedt,

Germany) oriented perpendicular to the femoral axis. The resulting slices were then ground to a thickness of 50–100 μm using a grinding-sliding machine (Microgrinding MG-4000; EXACT Cutting Systems).

The peri-implant new bone growth rate was initially analyzed using fluorescence microscopy (BZ-X 710; Keyence) and BZ-X image-analysis software (BZ-H4A; Keyence). The mineral apposition rate (MAR) was calculated by dividing the distance between fluorescent labels by the 7-d interval. The values were averaged from three slices of each specimen.

After getting the histomorphometric image, each section was stained with Stevenel's blue and Van Gieson's picrofuchsin, which resulted in the bright red staining of calcified bone and the blue staining of soft tissue. All images of the stained sections were obtained with a fluorescence microscope (BZ-X710; Keyence). The bone–implant contact (BIC) ratio was evaluated to quantify the ratio of bone directly in contact with the substrate to the substrate perimeter using Image J. The values were averaged from three slices of each specimen.

2.4 | Statistical Analysis

All data are expressed as mean (standard deviation). The statistical significance of differences among the four groups was analyzed using a two-way analysis of variance and Tukey's multiple comparison tests. A comparison of Sham and OVX+PSL to confirm osteoporosis was done using Student's t-test. The level of statistical significance was set at $P < 0.05$. The statistical analyses were conducted using the R software (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria).

3 | Results

3.1 | Surface Characterization

FE-SEM observation of the surface shows that a sub-micro-sized network structure was uniformly formed on the Ti surface after surface treatment (Figure 2a).

The results of the EDX analysis of the substrate are shown in Table 1. 1.8% of the elements in the surface layer of the Sr-Ti consisted of Sr.

The concentration of Sr ion released as a function of the square root of the soaking time in PBS from the samples subjected to Sr treatments is shown in Figure 2b. The Sr-Ti released 1.6 ppm of Sr ions within 1 h and then slowly released an additional 0.7 ppm over 14 d.

3.2 | In Vitro Study

3.2.1 | Cell Adhesion on SEM

The SEM images of the cells cultured on the substrates for 24 h are shown in Fig. 3a. The cells exhibited a well-spread morphology, displaying thick and polygonal cytoplasm with

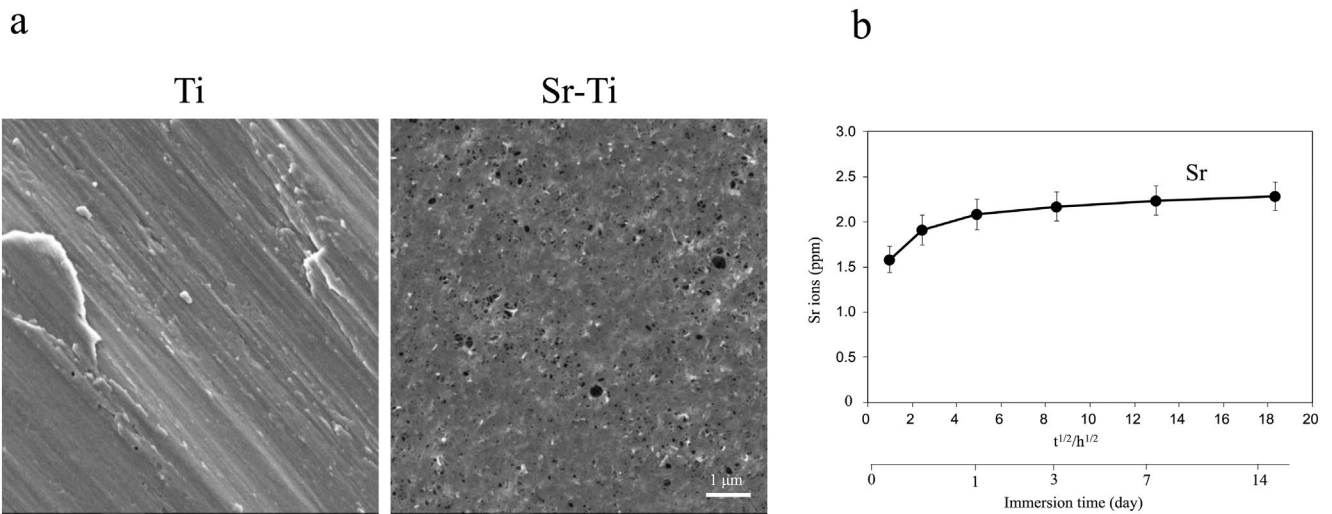


FIGURE 2 | (a) FE-SEM images of each surface of Ti and Sr-Ti metals. Scale bar: 1 μm . (b) Concentration of Sr ions released in PBS from the samples subjected to Sr treatments.

TABLE 1 | The results of EDX analysis of Sr treatment substrate.

Treatment	Element (at. %)			
	O	Ti	Ca	Sr
Sr	67.5	28.7	2.0	1.8

prominent pseudopodia on Ti, Sr, and TPTD-Ti groups. The cells in the TPTD-Sr group appeared to be centrally concentrated and elevated.

3.2.2 | Fluorescent Immunostaining of Actin Filament

The fluorescent immunostaining of actin filament images of the cells cultured on the substrates for 24h is shown in Figure 3b. The cells cultured on the Sr and TPTD-Ti groups exhibited extended filopodia relative to those cultured on the Ti groups. The cells on the TPTD-Sr group appeared to be centrally concentrated and elevated, similar to the SEM image, and the actin filaments ran vertically, reducing the cell area. Quantification of the aspect ratio showed that the TPTD-Sr group was significantly agglomerated with a smaller aspect ratio than the other groups (Figure 3c). Cell counts were not significantly different as in the proliferation assays described below (Figure 3d).

3.2.3 | Cell Proliferation

The XTT assay was employed to assess cell proliferation and viability in each group. No significant differences in cell viability were observed among the four groups at 1 and 7d (Figure 4a). The combination of Sr-Ti and TPTD demonstrated no evidence of cytotoxicity in comparison to the other groups.

3.2.4 | Alkaline Phosphatase Activity Assay

The ALP activity relative to the protein after 7d of culture in the TPTD-Sr group was significantly higher than that of the

TPTD-Ti group (Figure 4b). Sr-Ti tended to activate ALP activity, whereas TPTD treatment did not elicit this response.

3.2.5 | Alizarin Red Staining

Extracellular matrix mineralization after 21d of culture was detected by Alizarin Red staining. The graph and representative image are shown in Figure 4c. The TPTD-Sr group showed significantly higher absorbance spectra than the Ti, Sr, and TPTD-Ti groups.

3.3 | In Vivo Study

All animals had uneventful surgery. No wound infections or deaths were observed.

3.3.1 | Confirmation of Osteoporosis Induction

BMD was evaluated via micro-CT 8 weeks after OVX. The BMD of the OVX + PSL group was 46.63 (13.89) mg/cm^3 , which was significantly lower than that of the sham group (134.17 (45.72) mg/cm^3) ($p=0.03$) (Figure 5a).

3.3.2 | Biomechanical Testing

The bone-bonding strength was quantified through a push-out test. The maximum loads of Ti, Sr, TPTD-Ti, and TPTD-Sr groups at 6 weeks were 99.97 (42.54), 171.07 (43.93), 92.00 (56.83), and 269.92 (177.36) N, respectively. No significant difference was observed, whereas the TPTD-Sr group was the strongest, followed by the Sr group (Figure 5b). At 12 weeks, the maximum loads of Ti, Sr, TPTD-Ti, and TPTD-Sr groups were 90.83 (45.64), 155.72 (16.92), 133.20 (9.09), and 280.58 (18.48) N, respectively. The Sr group was significantly stronger than the Ti group ($p=0.02$), and the TPTD-Sr group was significantly stronger than the other Ti, Sr, and TPTD-Ti groups ($p<0.001$, Figure 5c).

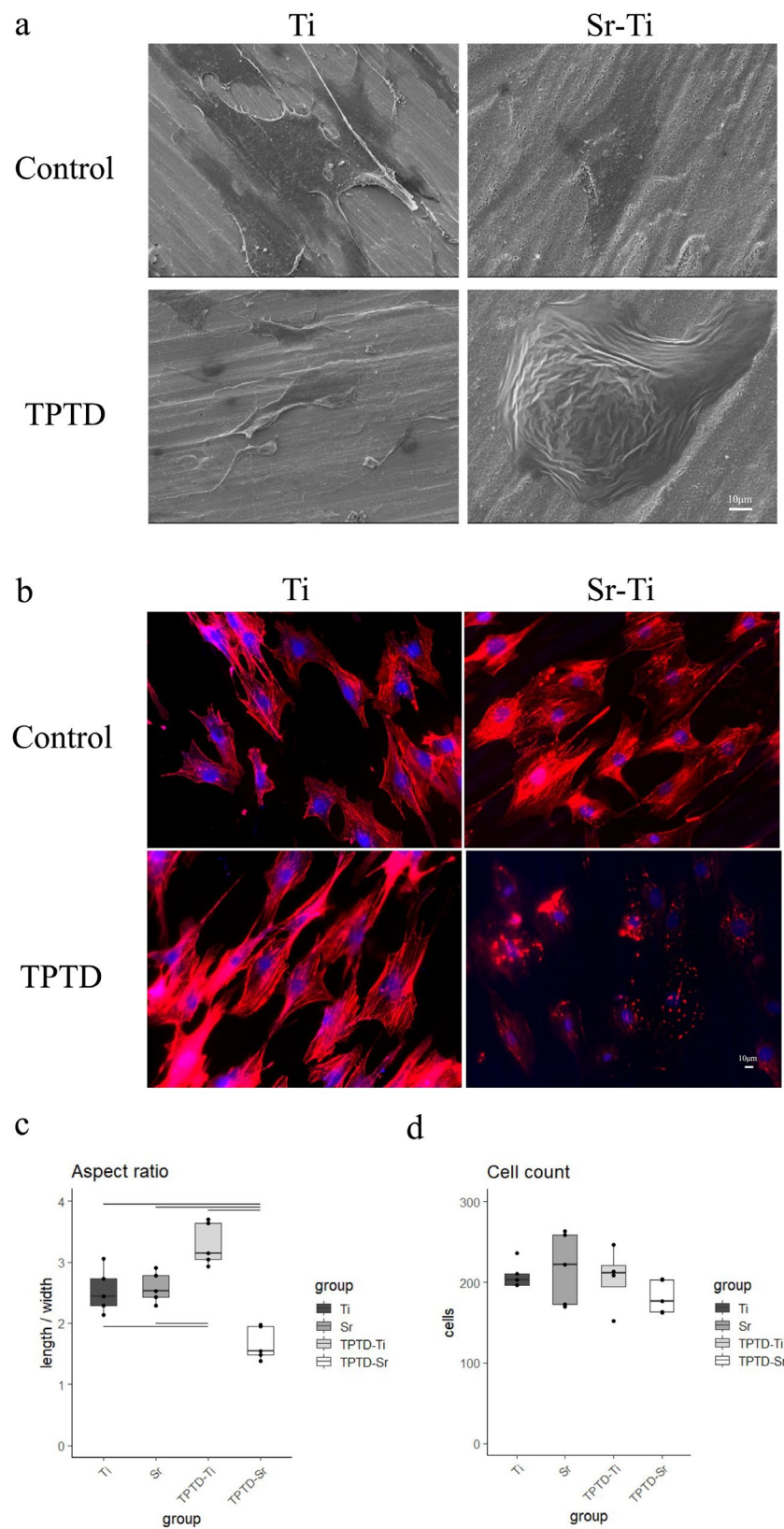


FIGURE 3 | (a) Cell attachment after 24 h incubation observed by SEM. Scale bar: 10 μ m. (b) Cell attachment after 24 h incubation on fluorescent immunostaining images (red: Actin filaments and blue: Nuclei). Scale bar: 10 μ m. (c) Length/width aspect ratio. Bars indicate $p < 0.05$ as per Tukey's multiple comparison test. (d) Cell count.

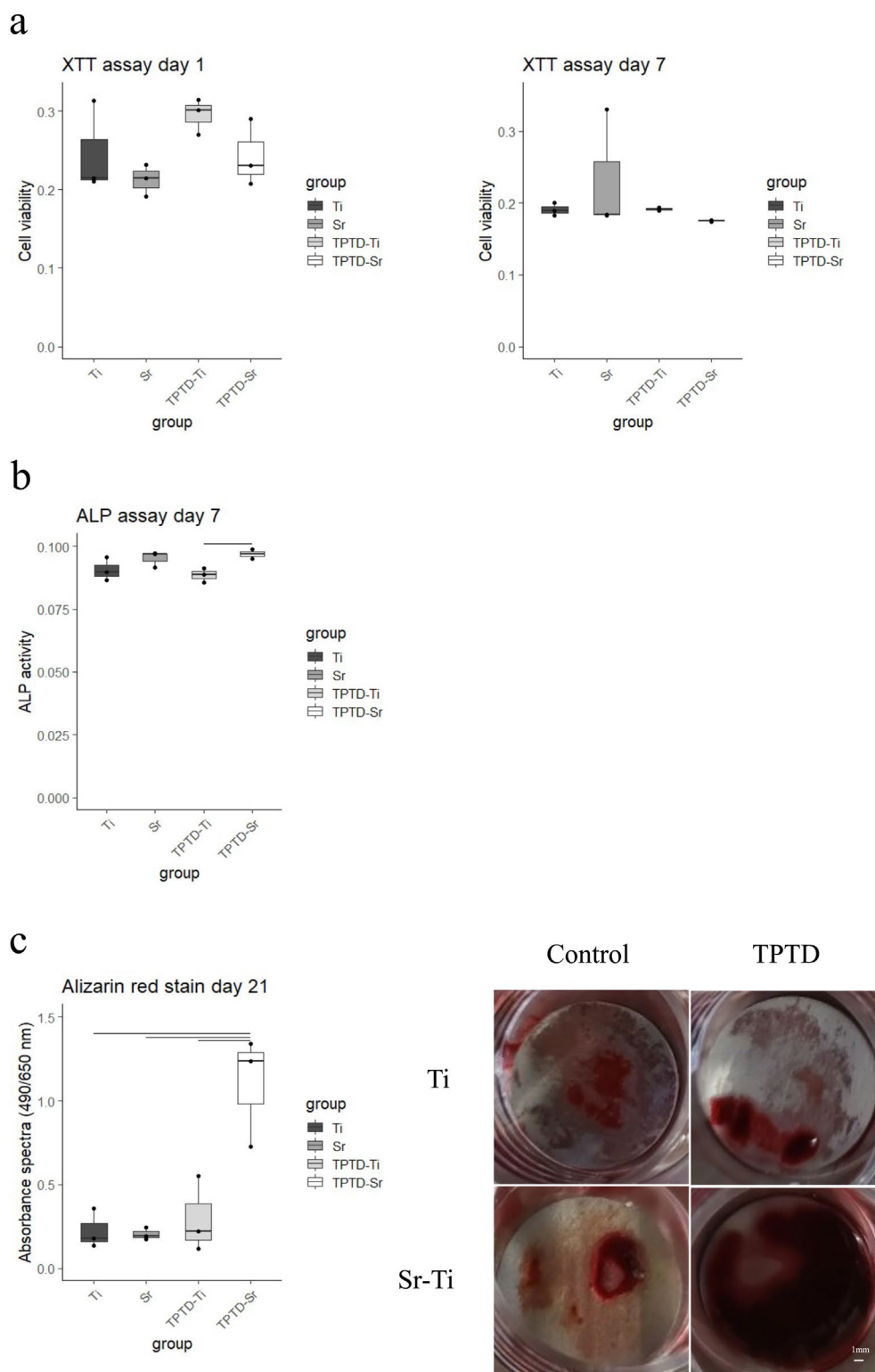


FIGURE 4 | (a) Cell proliferation and viability at 1 and 7 d were determined by the XTT assay ($n = 3$). (b) ALP activity after 7 d of incubation in each group ($n = 3$). (c) Mineralization of each sample measured by alizarin red staining ($n = 3$). Bars indicate $p < 0.05$ as per Tukey's multiple comparison test. On the right is a representative macro image. Scale bar: 1 mm.

3.3.3 | Radiological Analysis

Bone morphology measured by micro-CT was summarized in Table 2 and Figure 6. Tb. N and Tb. Sp significantly improved

by the TPTD-Sr group over the Ti group at 6 weeks ($p = 0.04$ and 0.03 , Figure 6c). No significant difference was found at 12 weeks, whereas bone morphology tended to improve when TPTD was injected (Figure 6d–f).

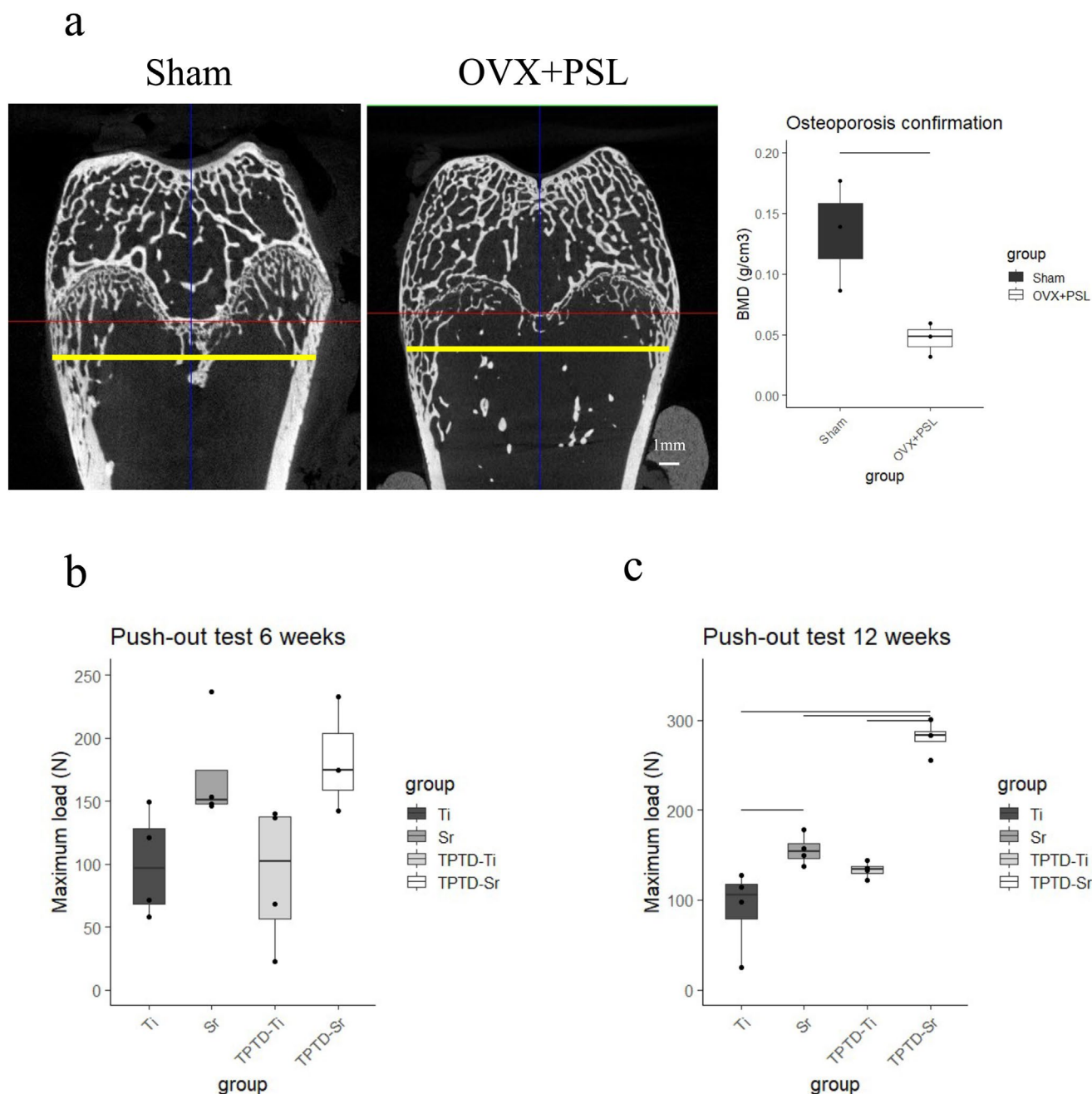


FIGURE 5 | (a) Micro-CT images to confirm osteoporotic condition. The bone mineral density was measured 3 mm proximal to the distal femoral epiphyseal line (yellow line). The graph illustrates a bone mineral density comparison ($n=3$). Bars indicate $p < 0.05$ by Student's t -test. Scale bar: 1 mm. Maximum load measured in the push-out test at (b) 6 weeks and (c) 12 weeks ($n=4$).

3.3.4 | Histology and Histomorphometry

Representative histological and histomorphometric images at 6 and 12 weeks are presented in Figure 7. TPTD groups generally exhibited improved bone morphometry, while Sr groups displayed stronger osseointegration. At 6 weeks MAR, the TPTD-Sr group was significantly larger than the Ti group ($p=0.01$, Figure 7a,c). At 12 weeks MAR, the Ti group was significantly smaller than other groups (vs Sr: $p=0.04$, vs. TPTD-Ti: $p=0.02$, vs. TPTD-Sr: $p=0.003$, Figure 7e,g). Regarding BIC at 6 weeks, the Sr group showed superior osseointegration to the Ti group ($p=0.008$). The TPTD-Sr group demonstrated greater osseointegration than

the Ti and TPTD-Ti groups ($p < 0.001$, $p=0.001$, respectively, Figure 7b,d). At 12 weeks BIC, the Sr group demonstrated superior osseointegration compared to the Ti group ($p=0.02$). The TPTD-Sr group demonstrated more osseointegration than the other three groups (vs Ti: $p < 0.001$, vs. Sr: $p=0.03$, vs. TPTD-Ti: $p=0.002$, Figure 7f,h).

4 | Discussion

The present study demonstrated that the combination of Sr-Ti implants with systemic TPTD administration enhanced osseointegration in osteoporotic conditions. This study's results showed that

TABLE 2 | Bone morphology measured by micro-CT at 6 and 12 weeks.

6 weeks				
Group	Ti	Sr	TPTD-Ti	TPTD-Sr
BV/TV (%)	29.27 (13.70)	40.34 (8.13)	38.54 (4.56)	47.13 (9.60)
Tb.N (1/mm)	4.37 (1.78) *	5.50 (1.05)	5.92 (0.49)	6.88 (0.87) *
Tb.Sp (um)	97.15 (11.21)	90.34 (9.36)	86.20 (4.34)	76.30 (9.20)
Tb.Th (um)	65.04 (7.43) **	73.78 (9.52)	65.25 (7.00)	68.15 (6.91) **
12 weeks				
Group	Ti	Sr	TPTD-Ti	TPTD-Sr
BV/TV (%)	30.33 (10.23)	39.09 (19.75)	49.31 (8.58)	41.71 (3.40)
Tb.N (1/mm)	4.26 (1.03)	5.11 (1.95)	6.52 (0.43)	5.46 (1.02)
Tb.Sp (um)	101.22 (4.93)	92.60 (15.02)	82.25 (4.32)	93.54 (9.80)
Tb.Th (um)	70.18 (6.13)	73.72 (12.55)	75.25 (8.45)	77.45 (8.16)

Note: * and ** indicate TPTD-Sr group of Tb. N and Tb. Sp significantly improved over Ti group at 6 weeks.

Abbreviations: BV/TV: percent bone volume, Tb.N: trabecular number, Tb.Sp: trabecular separation, Tb.Th: trabecular thickness.

the dual therapeutic approach produced superior outcomes compared to either treatment alone, as evidenced by increased biomechanical strength, enhanced MAR, and higher BIC in the *in vivo* study. The synergistic effects were in part notable in the *in vitro* studies, where the TPTD-Sr group exhibited markedly higher ALP activity and mineralization, suggesting enhanced osteoblastic differentiation through complementary cellular mechanisms.

It has been reported that nano/micro-roughness on the surface of implants induced by Sr-loading alkali and heat treatment can effectively improve the bioactivity of implants by imitating the actual bone structure [8, 9, 38]. Nano/micro topographies can enhance contact area and hydrophilicity, resulting in greater adsorption of extracellular matrix proteins and enhanced cell adhesion [39, 40]. Several other Sr-loaded implant studies have reported improved bioactivity both *in vitro* and *in vivo* [22, 41, 42]. The strength of our surface treatment method lies in its simplicity, being as straightforward as immersion and heat, and its affordability. It can be applied to a Ti surface regardless of its morphology, such as a porous structure, and allows for adjustment of the Sr concentration [13–15, 28]. Regarding the Sr release profile, our *in vitro* measurements were limited to 14 days. However, previous studies provide strong evidence for continued Sr release and biological activity beyond this timeframe. Okuzu et al. demonstrated that similar Sr-Ti implants maintained elevated serum Sr levels at 16 weeks post-implantation in rabbits, with implant-derived Sr detected in the peri-implant bone at 4 and 8 weeks. These implants showed superior bone-bonding strength and BIC compared to controls, with benefits maintained for up to 24 weeks [13]. Additionally, Honda et al. reported that Sr-loaded porous titanium implants in rabbit critical-sized defects demonstrated remarkable bone ingrowth and marrow regeneration over 12 months [15]. Supporting the technical feasibility of long-term ion release, Gallab et al. documented continued iodine release from similarly treated titanium surfaces for up to 90 days with sustained antibacterial efficacy. There is elution from the crystal surface within a few hours, followed by elution by diffusion from inside the crystal.

Experiments have shown that diffusion from within the crystal is small but persists for quite a long time [43]. These findings may suggest that the Sr-Ti implant maintains therapeutic Sr release well beyond our measured 14-day period, contributing to the observed long-term enhancements in osseointegration.

The effects of TPTD on promoting osseointegration have been reported at doses of 0.5–100 µg/kg, frequencies of 1,3,5,7 times per week, and animal species of mice, rats, rabbits, and dogs [18, 19, 21, 23–27, 44]. A few studies have shown that Ti implants loaded with functional substances, when combined with systemic TPTD administration, promote osseointegration. Li et al. reported that a combination of systemic TPTD and zoledronic acid-loaded Ti improved implant osseointegration more than either treatment alone [24]. Tao et al. presented the synergistic effect of TPTD and Sr-doped hydroxyapatite coating Ti on increasing osseointegration in a rat model [22]. In the present study, osseointegration is also facilitated by TPTD administration alone and synergistically by the combination of our Sr-loaded Ti implants and systemic TPTD treatment.

Systemic TPTD administration improves trabecular-bone microarchitecture by stimulating modeling-based bone formation [45]. In the present study, we confirmed significant improvement in bone morphology surrounding the implants through TPTD administration and combination with Sr-Ti. The bone morphology of the TPTD-Sr group improved at 6 weeks, as shown by the significant difference in Tb.Sp and Tb.N, and bone morphology tended to improve when TPTD was injected at 12 weeks. Treatment with TPTD and Sr-loaded Ti thickens trabecular bone as well as osseointegration and delays the failure of the bone-implant interface, as evidenced by the increasing trend of maximum load in the 6-week push-out test and the significant increase in the 12-week test [27].

Local or continuous administration of TPTD results in predominant bone resorption and a less osteogenesis-promoting effect [46, 47]. Therefore, the effect of TPTD loading on

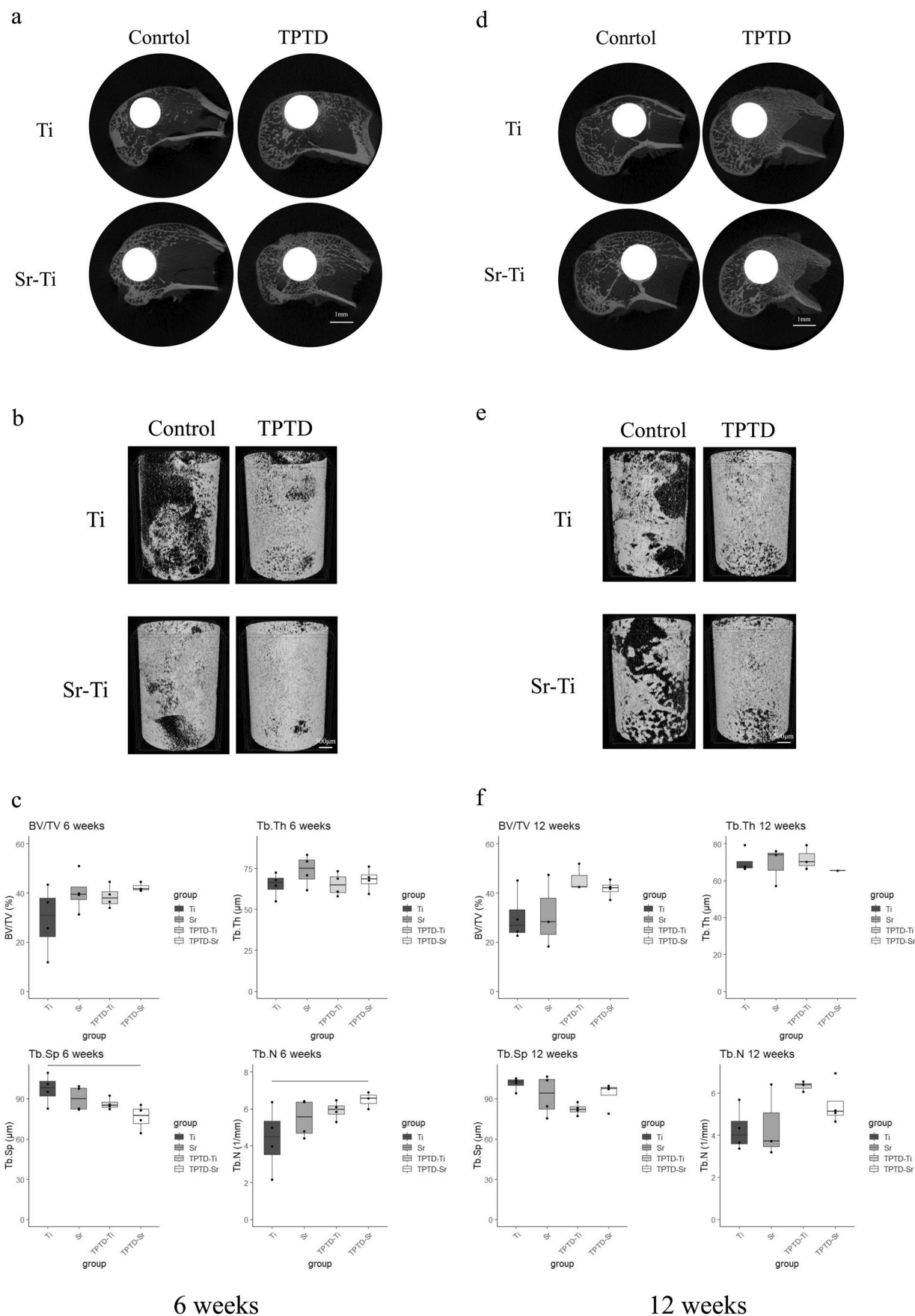


FIGURE 6 | Micro-CT images. 2D images of the plane perpendicular to the implant at (a) 6 weeks and (d) 12 weeks. Scale bar: 1 mm. 3D images of the 3–7 pixel region of interest around the implant at (b) 6 weeks and (e) 12 weeks. Scale bar: 500 μm. The graphs show the bone morphology of the 3D image at (c) 6 weeks and (f) 12 weeks. Bars indicate $p < 0.05$ as per Tukey's multiple comparison test.

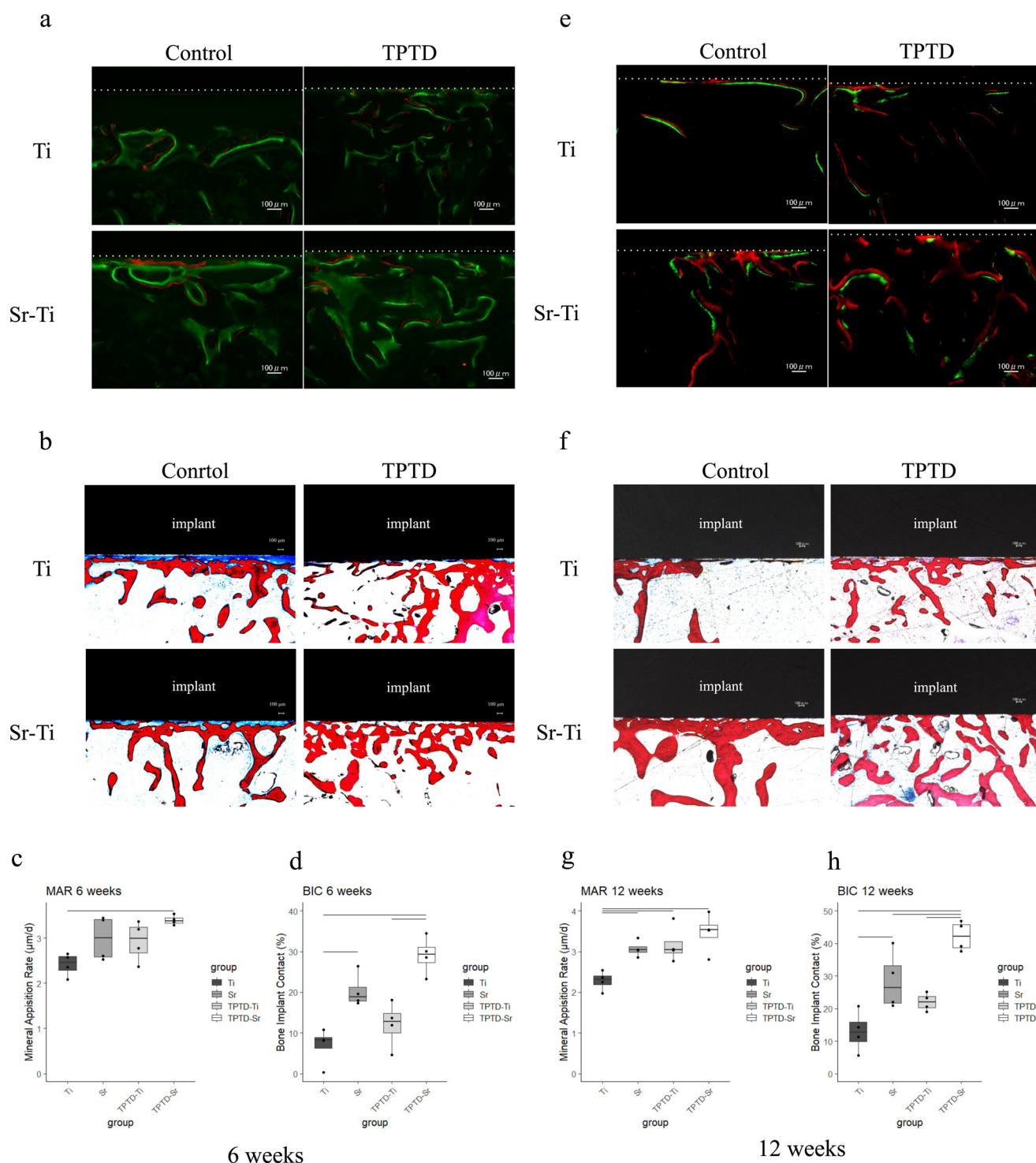


FIGURE 7 | Representative fluorescence-microscopy images (green: Calcein and red: Alizarin red) at (a) 6 weeks and (e) 12 weeks. The dotted line is the boundary between the implant and the bone. Scale bar: 100 μm . Mineral apposition rates (MAR) ($\mu\text{m/d}$) at (c) 6 weeks and (g) 12 weeks. Representative histological sections stained with Stevenel's blue and van Gieson's picrofuchsin (red: Bone and blue: Soft tissue) at (b) 6 weeks and (f) 12 weeks. Scale bar: 100 μm . Bone-implant contact (BIC) ratio (%) at (d) 6 weeks and (h) 12 weeks. Bars indicate $p < 0.05$ in Tukey's multiple comparison test.

implants is unlikely to be effective. A recent report showed that macroporous Ti-alloy scaffolds loaded with parathyroid hormone-related protein, synthesized by deriving 1–34 amino acids of parathyroid hormone with triple Glu at the C-terminus and a phosphorylated Ser at the N-terminus,

promoted osseointegration in a rat model [48]. However, in osteoporotic conditions, it would be beneficial to enhance bone density and morphology through systemic administration of TPTD and achieve a synergistic effect with another treatment for implants.

The observed increase in mineralization could be attributed to both direct and indirect mechanisms. The elevated ALP activity directly increases the local phosphate concentration, facilitating hydroxyapatite crystal formation. Additionally, Sr from the implant can be incorporated into the crystal lattice, potentially creating more favorable conditions for mineral deposition [11]. Actin staining and SEM images showed that the co-treatment of TPTD and Sr-Ti resulted in the aggregation of cells and a tendency for early differentiation. Dual stimulation of the Wnt/ β -catenin pathway [30, 49] and osteoblast stimulation through different receptor mechanisms (CaSR and PTH1R) [46, 50] may also enhance the production and organization of bone matrix proteins, creating an optimal microenvironment for mineralization. Regarding bone turnover, our dynamic histomorphometric analysis revealed increased MAR in the TPTD and/or Sr-treated groups, which directly reflects enhanced osteoblastic activity and a higher bone formation rate. Although it is reported that Sr suppresses bone turnover, whereas TPTD increases turnover [51]. This study's results demonstrated that the combined effect appears to favor enhanced bone formation, as evidenced by the improved BIC and trabecular parameters observed in our histological and micro-CT analyses. While we did not directly measure specific bone formation and resorption markers in this study, the structural and dynamic changes we observed are consistent with modulated bone metabolism that favors new bone formation. The net effect of this combination likely depends on the timing and dosing of each agent, which represents an important direction for future research.

The superior performance of the combinatorial approach can also be attributed to the complementary actions of local and systemic treatments. Sr released from the implant surface provides localized osteogenic stimulation, while systemic TPTD administration enhances overall bone metabolism. This dual-action strategy appears to create an optimal environment for peri-implant bone formation and remodeling.

The study's findings have significant clinical implications for improving implant outcomes in patients with osteoporosis. The combination of Sr-loaded implants with TPTD therapy could potentially reduce implant failure rates and accelerate osseointegration in challenging bone conditions. This approach may be particularly beneficial for older patients or those with severe osteoporosis who are at high risk for implant complications.

Limitations of this study are as follows: (1) The absence of alkali and heat-treated Ti controls without Sr loading. This makes it difficult to distinguish between the effects of Sr chemistry and changes in surface topography. Although our comparison of Sr-loaded and untreated Ti surfaces reveals clear differences, it is not possible to isolate these factors completely. Nevertheless, this approach is clinically relevant, as most commercial implants utilize untreated Ti surfaces, and our methodology aligns with established protocols in ion-loaded implant research [13, 52]. (2) The rabbit model is well-established for osteoporosis and TPTD research, but the bone metabolism and remodeling rates differ from those of humans. Further studies in larger animal models or clinical trials are needed to confirm

the translational potential of this approach. (3) TPTD affects bone formation depending on dose and frequency [45, 53, 54]. The TPTD dosage and the balance of Sr ions around the implant in vivo and in vitro may differ. Further, the dose of TPTD for animals is much higher than for humans. Therefore, different conditions could diminish the effectiveness of the combination. (4) The relatively small sample size in our animal experiments, while consistent with similar studies in the field and sufficient to achieve statistical significance, may limit the generalizability of this study's findings. Additionally, the long-term effects and safety of this combination treatment beyond 12 weeks were not assessed in this study. Future research with larger sample sizes should investigate the sustained benefits and potential adverse effects of prolonged exposure to both Sr and TPTD. (5) This study does not fully elucidate the underlying molecular pathways. It would provide more comprehensive insights to expand our investigation to include other relevant cell types, such as vascular cells and osteoclasts, and to conduct experiments that account for reactive oxygen species. Furthermore, in our follow-up studies, we plan to complement transcriptomic analyses with targeted validation using qPCR and Western blot to gain a better understanding of the molecular basis of the enhanced osteogenic properties observed with the Sr-Ti and TPTD combination.

5 | Conclusions

This study demonstrated that the combination of Sr-Ti implants and systemic TPTD administration improves bone quality surrounding the implants and synergistically enhances osseointegration in an osteoporotic environment. This novel approach shows promise for improving implant outcomes in patients with osteoporotic bone quality.

Acknowledgments

The electron microscopic study was conducted at the Division of Electron Microscopic Study, Center for Anatomical Studies, Graduate School of Medicine, Kyoto University.

Ethics Statement

This study was approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, Japan (approval number: MedKyo23566).

Conflicts of Interest

Asahi Kasei Pharma Corporation provided teriparatide acetate.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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