The effect of hydrofluoric acid treatment on titanium implant osseointegration in ovariectomized rats

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A R T I C L E   I N F O

Article history:
Received 8 December 2009
Accepted 8 January 2010
Available online 4 February 2010

Keywords:
Titanium
Fluoride
Surface modification
Osteoporosis
Osseointegration

A B S T R A C T

This study aimed to investigate the effects of hydrofluoric acid (HF) treatment of grit-blasted Ti implants on osseointegration in ovariectomized (OVX) rats. After blasting with aluminium oxide particles, half implants were treated with 0.2 vol.% HF, and the other half were kept non-modified as control. The topographical and chemical changes of implant surface were determined by Scanning Electron Microscope, Atomic Force Microscope, and X-ray Photoemission Spectroscopy. 12 Weeks after bilateral ovariectomy, each rat accepted two implants in distal femora, with the control implant on the left and the fluoride-modified on the right. As a result, fluoride modification induced markedly changed surface topography and chemical composition. 12 Weeks after implant insertion, the fluoride-modified implants showed improved osseointegration compared to control, with the bone area ratio and bone-to-implant contact increased by 0.9- and 1.4-fold in histomorphometry, the bone volume ratio and percent osseointegration by 0.8- and 1.3-fold in micro-CT evaluation, and the maximal push-out force and ultimate shear strength by 1.2- and 2.0-fold in biomechanical test. These promising results indicated that HF treatment of Ti surface improved implant osseointegration in OVX rats, and suggested the feasibility of using fluoride modification to improve Ti implant osseointegration in osteoporotic bone.

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1. Introduction

Titanium (Ti) has been widely used in dental and orthopedic implants due to its favorable biocompatibility [1]. When exposed in oxygen, a thin oxide layer would form on the surface spontaneously to prevent Ti from further corrosion and hinder metallic cation release after implantation in human body [2]. This oxide coat mainly consisting of titanium dioxide (TiO₂) is the substance that bone tissue is exposed to after implantation, and facilitates the biocompatibility of Ti implants [3]. Currently, osseointegration has been to the criteria for endosseous implant success, which is defined as the formation of a direct bone-to-implant interface observed at the light microscopic level [4]. Surface roughness of the implant has been demonstrated to influence the quality and rate of osseointegration [5,6], which may be explained by the enhanced contact area between bone and the implants or the increased ability to retain the initial blood clot [7]. Surface chemical composition of Ti also affects implant osseointegration. Previous reports indicate that fluoride ions directly stimulate osteoblast proliferation and function in vitro [8,9], and promotes new bone formation in vivo [10,11].

Different surface topography and chemical composition of Ti can be obtained by various progressing methods and influence tissue response to its surface [12,13]. Fluoride modification of Ti has been demonstrated to significantly enhance osteoblast functions and stimulate new bone formation on the bone–implant interface [14,15]. Recently, Ti implants with fluoride-rich chemical composition and specific micro/nano pattern topography have been shown linked to improved biocompatibility in vitro and enhanced implant osseointegration in vivo, with increased expression of osteogenic markers, peri-implant bone mineral density and reduced necrosis, gene expression of inflammatory and resorption markers [16–18]. On the other hand, preclinical and clinical evidence has indicated that osteoporosis can impede implant osseointegration and interventions to stimulate relatively decreased bone formation or inhibit excessive bone resorption on the bone–implant interface are essential [19–21]. Since fluoride-modified Ti has been shown linked to increased bone formation and possible decreased bone resorption, it promotes us to investigate the influences of fluoride modification of Ti implants on osseointegration in osteoporotic animals.

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The present study was designed to investigate the effects of diluted hydrofluoric acid (HF) treatment of grit-blasted Ti implants on osseointegration in ovariectomized (OVX) rats. After blasting, the fluoride-modified and non-modified Ti implants were inserted into the distal femoral metaphysis and medullary canal of the OVX rats. Before implant insertion, the topographical and chemical changes of implant surface were determined by Scanning Electron Microscope, Atomic Force Microscope, and X-ray Photoemission Spectroscopy. 12 Weeks after implantation, the distal femora with implants were harvested and evaluated by histological, microcomputed tomography (micro-CT) and biomechanical test.

2. Materials and methods

2.1. Implants and treatments

The rod-shaped implants (n = 44) included in this study, were made of commercially pure (cp) titanium and supplied by Dr. Liu (National Engineering Research Center for Biomaterials, Sichuan University). The implants with a diameter of 4 mm and a length of 10 mm were uniformly machined and grit-blasted with 25 μm aluminum oxide (Al2O3) particles. All the implants were then sequentially washed with NaOH at 40 vol.%, HNO3 at 50 vol.% and deionised water in an ultrasonic bath according to previous reports [16,18], and then sterilized in an autoclave. Subsequently, one half of the implants went through an additional surface modification procedure with 0.2 vol.5HF (300 μL PH = 2.1) for 90 s. Immediately after HF treatment, the implants were washed in autoclaved water for 10 s, lyophilized under aseptic conditions and packed in autoclaved containers. While the other half of the implants were kept non-modified as control group.

2.2. Surface characterization and chemical analysis

The topography of the control and fluoride-modified implant surface was characterized using a field emission Scanning Electron Microscope (SEM, Inspect F, FEI, Holland). The surface roughness of implants was measured by an Atomic Force Microscope (AFM, Nanoscope MultMode & Explore SPM, Vecco Instrument, US). The measurements by AFM were conducted in ambient air under tapping mode with a scan rate of 0.7016 Hz and a scan size of 5 x 5 μm. The Root Mean Square (RMS), Surface Area diff. and Z range were estimated with the aid of Nanoscope imaging software. 8 Different areas of the surface for the control and fluoride-modified implant were measured for statistical analysis.

2.3. Animals and surgical procedures

The animal experiments were conducted in accordance with international standards on animal welfare as well as being compliant with the Animal Research Committee of the University. 20 Female Sprague-Dawley rats aged approximately 3 months and weighing 210–230 g were included in this study. All the animals were fed a diet and tap water were permitted. After bilateral ovariecotmy, 12 weeks were allowed to pass before implantation for the establishment of standard osteoporotic animal model in OVX animals. Subsequently, all rats received two different implants in the distal femora as previous reports [22,23]. Briefly, general anesthesia was achieved by intraperitoneal injections of 10% chloral hydrate (3.3 ml/kg). All surgical procedures were performed under sterile conditions. A 10 mm longitudinal incision was made along the medial side of the knee joint, and the extensor mechanism with the patella was dissected laterally. With the knee in flexion, a 1.2-mm hole was made through the intercondylar notch with a rotary drill cooled with sterile saline solution. The control implant was then inserted into the medullary canal of the left femur via distal femoral metaphysis, and the fluoride-modified implant into that of the right femur until the implant end was below the articular surface. The patella was relocated and the extensor mechanism was reconstructed. Soft tissues were sutured and the animals received intramuscular antibiotic and analgesic injection for three post-operative days. All rats were allowed for free movement without any restriction. 12 Weeks after implantation surgery, all the animals were sacrificed and the distal femora with implants were harvested for the following evaluation.

2.4. Histological analysis

Immediately after sacrifice, half of the specimens in each group (n = 10/group) were dissected for undecalced histological sections. The femora with implants were maintained in a 4% neutral formalin buffered solution for 2 days. Subsequently, these femora were washed, dehydrated in graded ethanol (40–100%), and embedded in methy1methacrylate [Technovit 7200 VLC; Exact Apparaturbau, Nordenstedt, Germany] without decalcification [24]. Perpendicular to the femoral shaft, each specimen was sectioned in 100-μm-thick slices using a rotary diamond saw (SP1600, Leica, Germany) cooled with running water, ground to approximately 70 μm by Leica SP2600 (Germany), and then stained in 1% toluidine blue. The histomorphometry was performed on sections approximately 2 mm below the epiphyseal plate, using semi-automated digitizing image analyzer system, consisting of a Nikon ECLIPSE E600 stereomicroscope, a computer-coupled Nikon Digital Camera D5100 and NIS-Elements F.2.20 image software. Bone-to-implant contact (BC) was calculated as the linear percentage of the interface with direct bone-to-implant contact to total interface of the implant in the cancellous bone. Bone area ratio (BA) was measured as the area percentage of bone tissue to the whole area, which was defined as a ring extending 200 μm from the implant surface (Fig. 1A).

2.5. Micro-CT evaluation

At the time of sacrifice, the other half specimens (n = 10/group) were scanned on a µCT system (µ-CT 80 scanner Scanco Medical, Bassersdorf, Switzerland) and reconstructed with an isotropic voxel size of 10 μm. The scanning system was set to 70 kV, 114 μA, 700 ms integration time, for maximal signal-to-noise ratio and maximal X-ray transmission through the titanium implant. Multi-level thresholds procedure (threshold for bone = 205 and threshold for implant = 790) was applied to discriminate bone from other tissues [25]. The three-dimensional (3-D) images acquired from microtomographic slices were utilized for quantitative evaluation, with the constrained 3-D Gaussian filter (σ = 1.2, support = 1) for partly suppression of the noise in the volumes. The volume of interest (VOI) included the trabecular compartment around implant from 2.0 mm below the growth plate to distal 100 slices, which was defined as a ring with a radius of 200 μm from the implant surface (Fig. 1B). After segmentation, the bone volume per total volume (BV/TV), the mean trabecular thickness (B.Th), the mean trabecular number (B.N), the mean trabecular separation (B.Sp), and the mean connective density (Conn.D) was assessed within the VOI zone. The VOI was calculated as the ratio between bone and total voxels in direct contact with the implant [26].

2.6. Biomechanical test

Immediately after micro-CT evaluation, the specimens (n = 10/group) were evaluated by biomechanical push-out test using a universal material testing system (Instron 5566; Instron, Norwood, MA, USA) according previous reports [24,26]. About 3 mm of the implant end in the femur metaphysis was exposed by epiphyseal separation. A custom designed mould for each specimen was made out of self curing plastic, in order to maintain the downward compression to centre the implant and align it vertically. The push-out test was performed at a compression speed of 1 mm/min. Displacement versus force was recorded and used for the determination of the maximal push-out force and ultimate shear strength.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analyses were conducted using the statistics package SPSS 13.0 (SPSS, Chicago, IL, USA). Multiple comparisons between groups were performed using t-test. Differences were considered significant for p < 0.05 and highly significant for p < 0.01.

3. Results

3.1. Surface topography and chemical composition

The SEM and AFM examination clearly depicted the effects of HF treatment on surface topography of the Ti implant (Figs. 2 and 3). A strong difference was observed at the nano-level for the two different groups. In quantitative roughness analysis by AFM (Table 1), the fluoride-modified implant showed rougher surface than that of the control group, with the RMS increased by 2.4-fold (p < 0.01), the Z range by 0.9-fold (p < 0.05) and the surface area diff. by 2.7-fold (p < 0.01). XPS investigation of the implant surface within the first 10 nm revealed that both Ti and oxygen concentrations increased in the fluoride-modified group, while carbon content decreased (Table 2). The atomic percentage of fluorine was 1.88% for the fluoride-modified implant surface, while no fluorine could be detected in the control group. The Ti 2p binding energy was presented in Table 3, and no titanium species of TiO or TiO2 were detected.
3.2. Animals

All rats completed the 12-week healing period without clinical infection and other disorders. Specimens with implants were harvested for the following examinations.

3.3. Histological analysis

The undecalcified sections with implants showed the beneficial effects of fluoride modification on implant osseointegration and peri-implant bone mass (Fig. 4A). Results of histomorphometry were expressed as bone area ratio (BA) and bone-to-implant contact (BC) in

Fig. 1. Scheme of the region of interest for histomorphometry and micro-CT evaluation, which was defined as a ring extending 200 μm from the implant surface.

Fig. 2. SEM micrographs of surface topography of the control and fluoride-modified implants at different magnifications (the upper row, ×2000; the lower row, ×40,000).
Fig. 4B. 12 Weeks after implantation, fluoride-modified implants showed significantly increased histomorphometrical parameters compared to control, with the BA increased by 0.9-fold \( (p < 0.05) \) and the BC by 1.4-fold \( (p < 0.01) \). These results demonstrated that fluoride modification increased Ti implant osseointegration and promoted peri-implant new bone formation compared to control group.

3.4. Micro-CT evaluation

The 2-D micro-CT images of the coronal plane through the center of implant axis and the transverse plane about 2 mm below the epiphyseal plate were showed in Fig. 5, which clearly depicted the effects of fluoride-modified implants on osseointegration and peri-implant trabecular microstructure. Quantitative evaluation gave more detailed information on bone volume ratio (BV/TV), percentage osseointegration (%OI) and trabecular architecture around implants, which was shown in Table 4. Fluoride-modified implants increased BV/TV by 0.8-fold \( (p < 0.05) \) and %OI by 1.3-fold \( (p < 0.01) \). For trabecular structural feature, Tb.N and Conn.D were also increased markedly in fluoride-modified implants, by 52.0% and 44.9% respectively compared to control implants \( (p < 0.05) \). Although Tb.Th and Tb.Sp did not reach a statistically significant change, Tb.Th showed increased value and Tb.Sp showed decreased value compared to control implants. These results indicated that the most potent effect of fluoride modification was on implant osseointegration expressed as %OI.

3.5. Biomechanical test

Results of push-out test were expressed as the maximal push-out force and ultimate shear strength in Table 5. 12 Weeks after implantation, fluoride-modified implants showed significantly increased values of the two biomechanical parameters. The maximal push-out force were increased by 1.2-fold \( (p < 0.05) \) and the ultimate shear strength by 2.0-fold \( (p < 0.01) \) of the fluoride-modified implants compared to control. These results indicated the changes of mechanical characteristics on bone–implant interface after fluoride modification.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Surface roughness of the control and fluoride-modified implants determined by Atomic Force Microscope.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>RMS (nm)</td>
</tr>
<tr>
<td>Control</td>
<td>12.21 ± 2.12</td>
</tr>
<tr>
<td>Fluoride-modified</td>
<td>51.85 ± 5.74*</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD, \( n = 8 \)/group. \( ^* p < 0.05 \) and \( ^{**} p < 0.01 \) vs. control group (by t-test).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Elemental composition within the first 10 nm of the implant surface expressed as atomic percentage examined by X-ray Photoemission Spectroscopy.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>Ti</td>
</tr>
<tr>
<td>Control</td>
<td>9.00</td>
</tr>
<tr>
<td>Fluoride-modified</td>
<td>9.52</td>
</tr>
</tbody>
</table>
In the present study, c.p. Ti implant surface was modified with fluoride by immersion in a 0.2 vol.% HF solution for 90 s after blasting with 25 μm Al₂O₃ particles according to previous reports [16,18]. Surface topographical parameters in SEM and AFM examination showed increased surface roughness at the nano-level in the fluoride-modified implants, and surface chemical composition determined by XPS showed increased content of titanium, oxygen and fluorine, and decreased content of carbon, which was consistent with previous reports [15,16]. 12 Weeks after implant insertion in OVX rats, this study presented an improved osseointegration in the fluoride-modified implants compared to control, with the BA and BC increased by 0.9- and 1.4-fold in histomorphometry, and the maximal push-out force and ultimate shear strength increased by 1.2- and 2.0-fold in biomechanical test. Micro-CT parameters such as BV/TV, %OI, Tb.N and Conn.D also obtained increase to different extent.

Fluoride modification of Ti surface has been indicated to improve implant osseointegration in ulna or tibia of rabbit [27–29], tibia of rat [30], and mandible or tibia of dogs [31,32]. Furthermore, the addition of sodium fluoride to threaded implants made of cured polymethylmethacrylate resulted in increased area of bone in the threads six weeks after implant insertion in estrogen deficient New Zealand white rabbits [11]. However, no reports about the effects of the fluoride-modified Ti surface on implant osseointegration have been found in osteoporotic animals. Thus, this study might be the first report indicating that fluoride modification of blasted Ti surface could improve implant osseointegration in OVX rats, which was a widely used animal model of postmenopausal osteoporosis induced by estrogen deficiency and characterized by low bone mass and deterioration of bone microarchitecture [33]. However, since the main characteristic of osteoporosis was up-regulated bone turnover, with excessive bone resorption and relatively decreased bone formation, why fluoride modification of Ti surface could improve implant osseointegration in osteoporotic bone?

First, the topography and chemical composition changes after HF treatment could promote new bone formation on bone–implant interface. The topography of Ti surface at micro and nano-level has been reported to modulate differentiation, proliferation, gene expression of osteoblast and osteoblast-like cells [34–37]. Thus, the increase of surface roughness of implants at the nano-level observed in the present study, might contribute to the promoted new bone formation and then the improved implant osseointegration. On the other hand, changes of surface chemical composition also played an important role in the improved biocompatibility of implants. Compared to control, the fluoride-modified implants revealed increased content of titanium, oxygen and fluorine, and decreased content of carbon on Ti surface within 10 nm. The low hydrocarbon content, presence of fluoride, hydride and oxide on Ti surface by HF treatment have been speculated to contribute to improved biocompatibility in the study of Lamolle et al. [16]. In addition, fluoride used to be applied as anti-osteoporotic drug for decades, whereas high dose of fluoride was related to the formation of poorly mineralized osteoid and other side effects. But fluoride ions on Ti surface after HF treatment have been shown to increase osteoblast differentiation and gene expression, such as increased mRNA levels of runx2, alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin, collagen I and IGFB1 in fluoride-modified implants placed in the rabbit or rat tibia [16–18]. Moreover, the oxygen of phosphate in the tissue fluid has been supposed to replace the fluoride on Ti surface after implant...
insertion, and the phosphate becomes covalently bound to the titanium surface, which may induce increased bone formation [27]. On the other hand, inhibition of active bone resorption on implant surface, especially in osteoporotic animals, played an important role in implant success. Fluoride has been speculated to inhibit osteoclastic function at cellular level, but was lack of defined evidence. Recently published report of Monjo et al. [17] gave us some useful information about the biological mechanisms of the functional attachment of fluoride-modified Ti implants inserted in cortical bone by studying the association of pull-out test with gene expression of osteoblast, osteoclast and inflammation markers. As a result, lower lactate dehydrogenase (LDH) activity and TRAP mRNA levels were found in fluoride-modified implants than control 4 weeks after implantation. In addition, the observed lower LDH activity was well associated with lower gene expression of inflammatory cytokines (IL-6 and TNF-α) in the neighboring peri-implant cortical bone. These results possibly suggested decreased bone resorption at fluoride-modified implant interface compared to control. Furthermore, fluoride ions on implant surface might interact with the new formed hydroxyapatite crystals and lead to the formation of fluoridated

**Fig. 5.** 2-D micro-CT images of the distal femora with implants 12 weeks after implantation. Images of the upper row were transverse slices approximately 2 mm below the epiphyseal plate; and the lower row was coronary images through the central portion of the long axis of implants.

**Table 4** Quantitative results of the distal femora with implants by micro-CT evaluation within volume of interest 12 weeks after implantation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Control</th>
<th>Fluoride-modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>22.1 ± 2.3</td>
<td>40.3 ± 5.2*</td>
<td></td>
</tr>
<tr>
<td>%OI</td>
<td>20.4 ± 2.1</td>
<td>46.9 ± 5.3**</td>
<td></td>
</tr>
<tr>
<td>Tb.Th (μm)</td>
<td>76.5 ± 7.6</td>
<td>80.5 ± 8.8</td>
<td></td>
</tr>
<tr>
<td>Tb.N (mm⁻¹)</td>
<td>2.5 ± 0.3</td>
<td>3.8 ± 0.4*</td>
<td></td>
</tr>
<tr>
<td>Conn.D (mm⁻³)</td>
<td>23.6 ± 3.1</td>
<td>34.2 ± 4.0*</td>
<td></td>
</tr>
<tr>
<td>Tb.Sp (μm)</td>
<td>432.6 ± 48.9</td>
<td>417.2 ± 45.5</td>
<td></td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD, n = 10 specimens/group. *p < 0.05 and **p < 0.01 vs. control group (by t-test). BV/TV: ratio of bone tissue volume to total tissue volume; %OI: ratio between bone and total voxels in direct contact with the implant; Tb.Th: the mean trabecular thickness; Tb.N: the mean trabecular number; Conn.D: the mean connectivity density Tb.Sp: the mean trabecular separation.

**Table 5** Results of the biomechanical push-out test 12 weeks after implantation.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Maximal push-out force (N)</th>
<th>Ultimate shear strength (N/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.6 ± 3.5</td>
<td>1.63 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Fluoride-modified</td>
<td>71.7 ± 10.2*</td>
<td>4.87 ± 0.55**</td>
<td></td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD, n = 10 specimens/group. *p < 0.05 and **p < 0.01 vs. control group (by t-test).
hydroxypapitite, which was less soluble than hydroxypapitite and therefore more resistant to osteoclastic resorption. Interestingly, fluoride modification of Ti surface produced stronger effects on BC (1.4-fold), %OI (1.3-fold) and the maximal push-out force (1.2-fold). These results indicated that surface modification of Ti mainly influenced bone-implant contact, rather than peri-implant bone density, which was detected in the region within 200 μm from the implant surface in the present study. Previous studies using different surface modifications for Ti implant surface have shown similar results to that of the present study [38,39]. This phenomenon might be due to the dependence of the contact between modified implant surface and the surrounding tissues. Due to the lack of diffusing capacity like other cytokines and the contact between modified implant surface and the surrounding tissues, therefore more resistant to osteoclastic resorption.

5. Conclusions

This study indicated that fluoride modification of grit-blasted c.p. Ti surface improved implant osseointegration in OVX rats 12 weeks after implantation. After treatment with 0.2 vol.% HF for 90 s, the fluoride-modified implants showed increased surface roughness at the nano-level and changed surface chemical composition, with increased content of titanium, oxygen and fluorine, and decreased content of carbon than control implants. These topographical and chemical changes after fluoride modification contributed to the enhanced implant fixation in OVX rats. The results of this study suggested the feasibility of using fluoride modification to improve Ti implant osseointegration in osteoporotic bone.

Acknowledgements

This study was supported by a grant from National Science Funds for Distinguished Young Scholars (No. 30825040).

Appendix

Figures with essential colour discrimination. Figs. 1, 3 and 4 in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.01.028.

References


