Evaluation of the effect of glow plasma nitriding of commercially pure titanium dental-implant on osseo integration through mechanical and histomorphometric analysis

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Abstract

Background: Oral implantology provides a reliable and rather safe solution to replace missing teeth. An implant is defined as a biomaterial which is embedded, either incompletely or totally, into the body for restorative or prosthetic purposes. The aim of current study was to evaluate the effect of plasma nitride treatment of commercially pure titanium dental implant on bond strength at the bone-implant interface by torque removal test and histomorphometric analysis after 2 and 6 weeks in comparison to non-treated one. Methods: Commercially pure titanium plates and screws were plasma nitride treated for 10 hours using glow plasma nitride apparatus. X-ray diffraction analysis and scanning electron microscope examination were carried out on the nitride surfaces of the plates. The femur of 10 white New Zealand rabbits was chosen as implantation sites. The femur of each rabbit received 2 screws and a total of 40 screws were implanted. For each period of time, 18 screws were tested for the torque required to remove the implant from the bone and 2 screws were kept for histological examination. Results: There was a significant increase in the bond strength and the new bone formation ratio of bone-implant interface with time. The torque removal forces for plasma nitride implants after 2 and 6 weeks were 30.22, 59.56 N.cm respectively and for non-treated implants after 2 and 6 weeks were 23.61 and 56.11N.cm, respectively. The new bone formation ratio for non-treated implants after 2 and 6 weeks were 1.56 and 3.04, respectively, and for plasma nitride treated after 2 and 6 weeks were 3.36 and 4.72, respectively. Conclusion: Plasma nitride treatment of commercially pure titanium plate screws significantly increased the torque removal value and new bone formation ratio at 2 and 6 weeks compared with the non-treated implant.

Keywords: Dental implant, plasma nitride, histomorphometric analysis, torque removal, osseo integration


Introduction

Oral implantology provides a reliable and rather safe solution to replace missing teeth (¹). An implant is defined as a biomaterial which is embedded, either incompletely or totally, into the body for restorative or prosthetic purposes (²). Following insertion, they incorporate with the bone after some time and act as an anchor
for the dental prosthesis, crown, bridge and overdenture. Therefore, they are considered as a substitute for the root of the missing tooth (3). The implant becomes one of the most promising fields in dentistry for improved human quality of life (4). Currently, dental implant treatment is considerably developed than it was before and a lot of its clinical achievement is associated with an improvement in biological responses and surgical management (5). Dental implants vary in material, dimensions and surface properties (6). Titanium demonstrates an ideal mix of characteristic properties for the manufacture of dental implants such as low weight, low modulus of elasticity, easy surface treatment and excellent biocompatibility (7). Accelerating the osseo integration of commercially pure titanium plates (CpTi) results in reducing the non-functional time period of the implant, increase its applicability in alveolar bone with low quality, minimum discomfort to the patient and minimize the failure rates (8). One of the methods used in accelerating the osseo integration process is increasing roughness. Rough surface characteristics play an important role in cell adhesion since they provide the necessary adhesion conditions such as surface area and contact point to adsorb and help in the cell adhesion process (9). In order to increase the rate of osseo integration, surface topographies of artificial materials should be comparable as possible in diameter and shape to the bone. The topographies of the implant surface are characterized at different size levels. At a macroscopic level, the thread shape and the pitch distance of the implant design are essential for the implants stability through high rate of osseointegration (10). At a microscopic level, surface roughness seems to guide cell adhesion in a differentiated manner. Round and/or elongated cells were observed interacting with and spreading themselves over surface irregularities (11). Plasma nitriding can be used for changing surface topography, to increase surface roughness and, consequently, higher surface wettability (12). Chemically and physically reactive plasma discharges are widely used to modify the surface properties of materials (13). Glow plasma nitride (PN) treatment constitutes a simple, dry technique, does not harm the environment, cost-effective, does not comprise the intrinsic properties of the biomaterial and affecting only its surface (14).

The aim of current study was to evaluate the effect of plasma nitride treatment of commercially pure titanium dental implant on bond strength at the bone-implant interface by torque removal test and histomorphometric analysis after 2 and 6 weeks in comparison to non-treated one.

Materials and Methods

Samples preparation

Three CpTi specimens were prepared (10*10mm) and thickness of 1mm which were cut from CpTi sheet using a bench nibbling machine (TAURUS, 7000-W6 CNC, Italy). All specimens were abraded successively by employing Sic grinding paper with various grits size (80, 120, 230, 400, 600, 800 and 1000) to acquire a flat grit and scratch-free surface and then polished with diamond suspension (15, 9, 6, 3, 1)µm for a smooth and mirror polished surface. Specimens were mounted by using mixing powder and liquid of a fast cold set material and the mold was up to 20mm thickness. Finally, in order to achieve a clean and polished surface, the specimens were cleaned through using ultrasonic and ethanol.

Plasma nitride

The nitriding procedure was performed in a D.C. glow discharge plasma system of a target cathode and anode disk of stainless steel. This is a low-pressure gas discharge unit consists of a vacuum chamber with two parallel electrodes. The cathode faced the anode, which provides an electric field for the gas to be discharged. The bottom shaft of the cathode electrode is shielded by insulator disk (ceramics). The top of the cathode is shielded by cathode space assembly which included ceramics insulator and stainless steel holder. The clean samples were placed on the cathode in the center of the dark shield. The cathode was connected to the D.C. power supply while the grounded chamber served as the anode. The nitrogen gas was discharged at 3mbar pressure, a voltage of 650V and a current of 0.02mA for glow plasma nitriding. The nitriding was carried out at various times (10 and 20 hours). The plasma chamber was evacuated to a vacuum pressure of (5x10^{-4} mbar), in order to ensure the complete removal of the heavy gases like hydrocarbons. All the samples were cleaned by argon plasma
sputtering for 15min prior to the plasma nitriding process. The nitriding gas was introduced in the evacuated chamber and the flow rate was adjusted until the pressure was stabilized at $2 \times 10^{-1}$ mbar. The high voltage D.C. power supply was 4kV; whereas the cathode voltage and discharge current were increased until the plasma was generated. After the process had finished, nitrogen was drawn from the vacuum chamber. The samples were kept until the ambient temperature in the vacuum chamber was reached. All nitride samples were kept in vacuum disector to protect them from the atmosphere until the analysis was carried out.

**X-Ray Diffraction Phase Analysis (XRD)**

Phase distribution of the untreated and plasma nitrified CpTi was examined using X-ray diffraction facilities (XRD; Shimadzu 6000, Japan) using Cu Kα radiation. XRD analysis was performed at room temperature in the 2θ range from 30˚- 80˚ with a 0.05˚ step and counting time of 5sec per step. The indexing of data and diffraction peaks were identified according to powder diffraction files (PDF), received from Intimations’ Center for Diffraction Data.

**Scanning electron microscope (SEM)**

The untreated and plasma nitride CpTi samples for 10 and 20 hours were examined by scanning electron microscope (JEOL-JSM-5600) to study the changes occurs on the sub-layer during the nitriding process. Samples for SEM were prepared in cross-sections to discover layers of the modified surface. The two nitride samples (10 and 20 hours) were mounted in cold-set mounting and gold-plated from both sides to make the sample holder electrically conductive before entering the samples into the SEM chamber.

**Contact angle measurement**

Commercially pure Titanium (grade 2) plates 10*10*1mm were used for pilot study by dropping equal amounts of normal saline (0.25ml) from a graduated container on each plate treated with plasma nitride for 10 and 20 hours. The contact angle formed between the titanium disc surface and a drop of normal saline was measured; the same test was done with a drop of blood (rabbit blood) instead of normal saline. Ten hours was the best-achieved wettability, therefore, it was selected for the study.

**In Vivo / Implantation and torque removal procedures**

Ten white New Zealand rabbits were divided into 2 groups for each healing intervals (2 and 6 weeks) each one consisted of 5 animals; one of them was sacrificed for histological study while the other 4 were sacrificed for the mechanical test by torque removal test. All instruments and towels were autoclaved at 134°C and 15bars for 90 minutes. Each animal was weighed before the operation to determine the required dose of anesthesia and antibiotic. Anesthesia was induced by intramuscular injection of ketamine hydrochloride (1ml/kg body weight) and xylcocaine 2% (1ml/kg body weight). Both femurs were shaved using shaving spray from outer side, and skin was cleaned with ethanol. The surgical towels were placed around the operation site and the surgery was performed under aseptic conditions. Later on, the incision was made on the lateral side to expose the distal side of the femur, the skin and fascia flap were reflected. Four implants, 2 were non-treated and 2 were plasma nitride-treated, implanted in the femur and each femur received one non-treated implant and one plasma nitride-treated implant. First whole preparation was done by drilling to prepare implant bed with cooling for first implant screw (Biohorizen, USA) insertion. Second whole preparation and drilling were similarly performed to insert the second implant. Both screws (non-treated and treated) were placed in the prepared wholes (proximal for non-treated and distal for treated implants) using screwdriver attached to an angle handpiece that fits the screw slit until 5mm (thread surface) of the screw was completely inserted in the whole with a bicortical penetration. The insertion torque was about 35-40N.cm using surgical engine (angle handpiece W&H, Austria). For more accuracy CT scan (LightSpeed VCT. France) and periapical X-ray (MyRay, Italy) were taken to determine the fitness of the implant in the bone, to check the implant apical area and to determine the bicortical bone penetration in the femur bone of rabbit for both implants.

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after surgery and before suturing. Muscle suturing was done with absorbable catgut suture 3/0 followed by skin suture with silk suture 3/0. The operation site was sprayed with a local antibiotic (oxytetracycline spray). Then long-acting systemic antibiotic (oxytetracycline 20%, 0.5ml/kg body weight) was followed for each rabbit.

After 2 and 6 weeks of healing periods, 4 animals from each group were used for mechanical testing by removal torque. The test was made up while the animal was anesthetized in the same manner and similar to that was used in the implant insertion procedure. The incision was made at the lateral side of femur then fascia and muscles were reflected to expose the implants screw. After that, the muscles were removed to expose the entire femur. The femur was supported firmly while performing the mechanical test to prevent any movement which may affect the accuracy of the test or fracture of the bone. A torque removal test was done by engaging the head of digital torque meter (angle handpiece W&H, Austria) into the slot in the head of the implant with anti-clockwise engine rotation and increase the torque gradually to determine the peak torque necessary to loosen the implant from the bone bed in N.cm.

In Vitro Histological testing

One animal from each group was used for histological testing. A surgical disc and mandrel (Komet Dental, Germany) were utilized for cutting the bone with high rotating speed. Auto cooling system straight surgical handpiece engine (NSK, Japan) was employed for cutting the bone around the implant about 10mm away from the head of the implant to prepare a bone-implant block for histological study. Bone-implant blocks were immediately stored in 10% freshly prepared formalin and left for overnight fixation, thereafter; bone with its implant placed in 10% solution of nitric acid for decalcification (3-5 days). After complete decalcification, the bone-implant block was divided horizontally (cross-section) into two parts using a sharp scalpel (DenTag, Italy). The specimen then passed in the process of histopathological preparation of slides. The specimens were molded in the center of paraffin block and adjusted to microtome where serial sectioning with 3-4μm of thickness for each section was performed and placed on a slide. Total of 25 sections were made for each treated and non-treated implant in each period of time (2 and 6 weeks). The slides were prepared for staining procedure on hematoxylin and eosin stains by using a routine staining technique. For microscopic examination, 3 photographs of each section were taken at 10, 20 and 40 magnifications by light microscope (Olympus BX51, DPS 72 camera). The area of new bone was marked according to criteria stated by Shapiro (2008)(15), in which coarse meshwork (trabecular bone) of pink tissue surrounding patches of much lighter (unstained tissue) or matrix. Osteoblast appears as a mononuclear cell with prominent nucleoli and deeply stained cytoplasm. They displayed in well-polarized fashion only along the woven bone surface and aggregated into a single layer of cells lying in opposition to new bone. Mature lamellar bone is less cellular than woven bone. The new bone formation area was measured using computer software (OUTO CAD, 2014). The ratio of new peri-implant bone formation (NBFR) was calculated using the following formula; NBFR = area of newly formed bone / total tissue area (16).

Data were analyzed using SPSS (Version 20; IBM Corp., Armonk, NY, USA) with P<0.05 significance level. Mann–Whitney t-test was used to analyze the difference of the removal torque values (N.cm) in the mechanical testing and for the NBFR analysis between the two groups at 2 and 6 weeks intervals while paired t-test was used to compare intervals within groups.

Results

X-ray diffraction analysis

Figure (1) displayed the XRD patterns of CpTi before and after plasma nitriding for different periods. The diffraction peaks of the untreated CpTi were found to be corresponding to 100, 002, 101, 102, 110, 103, 112 and 211° of Ti at 20 values 35.8°, 38.4°, 40.5°, 53.7°, 62.8°, 71°, 76.5° and 70.3°. For the CpTi-treated for 10 and 20 hours, the patterns showed the formation of (004) and (303) Ti2N at 20 values of 40.9° and 67.3°. XRD patterns also showed the formation of another nitride phase at 20 (35.2°) and (77.3°), which are representing the (012)
and (027) \( \zeta \) Ti4N3-x phase. The appearance of the reflection at 20 value of (38.5°) suggesting the formation of (104) \( \eta \) Ti3N2-x.

**Morphology of nitride layer by SEM**

The cross-sectional morphology of CpTi was observed using SEM and the thickness of the nitride layer formed on the surface of samples was evaluated. Figure (2) showed the cross-sections of CpTi plasma nitrided samples for 10 and 20 hours. The thickness of the nitride layers was quite uniform and there was no gap between the nitride layer and the metal substrate. Additionally, the thickness of the nitride layer formed on CpTi after 20 hours nitriding was more than that formed after 10 hours. The micrographs also showed that there were two zones in the nitried layer, a thin nitride film and a thick heat-affected zone developed during the plasma nitriding process.

**Mechanical testing**

A highly significant torque value was needed to remove the implants modified with plasma nitride after 2 weeks of implantation (30.22±1.18N.cm) compared to the torque value needed to remove non-treated implants (23.61±1.34N.cm) as shown in Table (1). Similarly, a highly significant torque value was needed to remove the implants modified with PN after 6 weeks of implantation (59.56±1.76N.cm) compared to the torque value needed to remove non-treated implants (56.11±2.36N.cm). Within groups, it was obvious that the torque value needed to remove implants from the bone bed was increased as healing periods increased (Table 1).

**Histomorphometric analysis**

The histological feature of the non-treated implants in rabbit femur after 2 weeks of implantation displayed osteoid tissue with several bone cells bounded by many new established capillaries while new bone trabeculae lined by osteoblast cell arranged as a rim of cells on the bone surface with active osteoid tissue, was the histological feature of the non-treated implants in rabbit femur after 6 weeks. The NBFR was significantly higher for the plasma nitrided treated implant at 2 weeks (3.36±0.48) than the non-treated implants at 2 weeks (1.56±0.38) as illustrated in Figure (3) and Table (2).

Microscopic views of the non-treated implants in rabbit femur after 6 weeks of implantation showed new bone formation with osteocyte cells. Other higher magnification view displayed osteocyte cell irregularly distributed in thick trabeculae and a large number of osteoblasts were observed. On the other hand, after 6 weeks of implantation, microscopic views for the section of the rabbit femur bones surrounding the implant-treated with plasma nitride showed an active process of bone development, designated by the active and large numbers of osteocytes clearly appeared. The plasma nitrided implant at 6 weeks after implantation showed significantly higher NBFR (4.72±0.52) than the non-treated implant (3.04±0.42) as shown in Figure (4) and Table (2). Within groups, the 6 weeks interval showed a significantly higher rate of NBFR compared with the 2 weeks interval for both treated and non-treated implant (Table 2).

**Discussion**

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There were numerous diffraction peaks observed in this study due to different times of exposure to plasma nitride as shown by the XRD analysis. Also, it was evident from the SEM that the surface of the specimen was well-covered with the nitriding layer. The nitriding time of 20 hours showed more phase formation than 10 hours and this may be due to the increase in the volume fraction to the nitrogen diffused in the CpTi substrate. The nitride layer formed on the surface of the implant gives rise to the development of roughness, grooves and extra topographical features which can be substantial for bone attachment and growth (17). The nitride layer formed on CpTi treated for 20 hours was thicker than CpTi treated for 10 hours; this was in line with findings of a previous study (18) in which plasma nitried samples of grade-5 titanium for 24 hours led to a significant increase in their wear-resistance compared to the non-treated samples due to increased thickness.

Increasing the wettability can lead to converting Ti surface from hydrophobic to hydrophilic surface which may accelerate osseointegration (19). Therefore, the best wettability achieved for the samples (10 hours) had been suggested as one of the factors that cause higher torque removal value and higher new bone formation ratio for the nitrided group than the non-treated implant group.

The histomorphometric investigation is an invasive technique; it is generally utilized as a quantitative technique for determining the level of bone to implant interaction. Characteristic parameters measured comprise the ratio of bone contact, the bone area within the threads and the number of osteocytes can be counted as well (20). Implants modified with PN in this study showed significantly more bone formation ratio than the non-treated implants at 2 weeks which could be attributed to the activation of osteoblasts by the nitride layer, the effect of wettability and roughness. Therefore, a higher number of osteoblasts, more bone trabeculae and active osteoid tissue were noticed in the nitride group. Also, it was observed that bone formation ratio at 6 weeks was higher in PN implant compared with non-treated implants at 2 weeks and, at the same time, the histological feature showed more bone formation ratio for the treated compared to the non-treated PN implants at 6 weeks. Osteoblasts and osteocytes were more around PN implant which means more formation and maturation of bone and more transformation of osteoblasts to osteocytes. Advanced bone formation rate at 6 weeks could be attributed to the early activation of the nitride implant to the tissue at the interface, as there was more new bone formed at 2 weeks than non-treated implants. Rough surface, increase wettability, nitrogen ion present at the interface could be the reason for the activation which could have a continuous effect up to 6 weeks and maybe more (21). Hydrophilic surfaces are preferred for blood coagulation over hydrophobic surfaces, thus, dental implants have been established with high hydrophilic and rough surfaces. Adsorption of proteins, for instance, fibronectin and vitronectin on the surface of dental implants could provoke cell adhesion and osseointegration (22). By the time, the formation of bone was increased for both PN treated and non-treated implant. The early high effect of nitride layer on osteoblast activity and bone formation from the first 2 weeks is desirable as the goal of osseointegration is achieved in the shortest possible healing time (23). For a non-treated implant, the difference in NBFR from 2 weeks to 6 weeks was high which indicated delay activation and bone formation while for the nitride treated surface the difference between 2 and 6 weeks was low which indicated early and continuous activation and new bone formation through this period. The normal physiological process of healing which increased by time could also be the reason as the longer the healing period, the more bone formation.

Removal torque has been used as a biomechanical measure of anchorage or endosseous integration, and has been used in several clinical and experimental investigations. Greater torque required to remove an implant may be interpreted due to the increase in strength of bony integration (24). The nitride-treated CpTi screw implants placed in rabbit bone recorded higher mean of removal torque value than non-treated screws at 2 weeks and 6 weeks of implantation. This indicated increased bone formation and bond strength at the bone-implant interface in the nitride-modified implants. High wettability of the nitride surface might accelerate bone formation more than non-modified screws. Plasma nitride treatment changes local chemical properties of the surface by formation of different phases which may lead to faster osseointegration process. Nitriding the surface and creating different nitride phases may provide a source of nitrogen ions which may help in the supplement of tissue protein with an essential component which is nitrogen and may help in increasing protein production for cell proliferation and so higher osseointegration takes place (25,26).
Besides wettability, rough surfaces provoke an anchorage of proteins and consequently provide better adhesion of osteoblastic cells. It was found that the rough surfaces would support the growth of osteoblasts and lead to new bone formation, this agreed with findings of previous studies \(^{17,27}\).

However, different mean torque values were recorded by other studies. For example, \(^{28}\) and \(^{29}\) recorded 12.375N.cm and 17.87N.cm removal torque values, respectively, for the same implantation periods but with hydroxyapatite coating. The variation in values may be due to differences in material types, mode of action for each material used and primary stability which are either uni- or bi-cortical.

Furthermore, both treated and non-treated implants showed increased removal torque value between 2 and 6 weeks of implantation which was statistically significant. Increase in osseointegration of implants as time proceeded might reflect changes that occur in the bone structure around implants in addition to the increase in bone maturity with time as normal healing process \(^{30}\).

This study was aimed to evaluate the effect of plasma nitride treatment of screw-shaped commercially pure titanium dental implant after 2 and 6 weeks in comparison to non-treated one. However, studying the effect of plasma nitride on osseointegration at early and late periods (1 week and 8 weeks) and using other types of gases (nitrogen with hydrogen) could have differential results and are suggested for future studies. Since only one animal was sacrificed for histological study in each group, the number of animals in this study considered as a limitation.

**Conclusion**

Within the limitations of this study, we concluded that increasing wettability for the 10 hours increased the torque removal value and new bone formation ratio at 2 and 6 weeks of plasma nitriding CpTi screws compared to non-treated screws. Moreover, there was an increase in new bone formation ratio with time for both treated and non-treated CpTi implants screws.

**Ethical Clearance**

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

**Conflict of Interest**

The authors declare that they have no conflict of interest

**Funding:** Self-funding.

**Table (1) The torque removal in different groups and durations**

<table>
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<th>Groups</th>
<th>No.</th>
<th>(P)-value</th>
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<td>2 weeks</td>
<td>Non-treated mean(SD)</td>
<td>18</td>
<td>0.000*</td>
</tr>
<tr>
<td>6 weeks</td>
<td>Treated with plasma nitrating mean(SD)</td>
<td>18</td>
<td>0.000*</td>
</tr>
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</table>

* indicated significant differences between groups \((P<0.05)\) using Mann–Whitney \(t\)-test. The same superscript * vertically within groups indicated a significant difference \((P<0.05)\) using paired \(t\)-test.

**Table (2) The new bone formation ratio in different groups and durations**
<table>
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<th>Group</th>
<th>No.</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Non-treated mean(SD)</td>
<td></td>
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<tr>
<td>2 weeks</td>
<td>Treated with plasma nitrating mean(SD)</td>
<td></td>
<td></td>
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<tr>
<td>1.56(0.38)</td>
<td>3.36(0.48) *</td>
<td>50</td>
<td>0.000*</td>
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<tr>
<td>6 weeks</td>
<td>3.04(0.42) *</td>
<td>4.72(0.52) *</td>
<td>50</td>
</tr>
</tbody>
</table>

* indicated significant differences between groups (P<0.05) using Mann–Whitney t-test.

The same superscript * vertically within groups indicated a significant difference (P<0.05) using paired t-test.

Figure (1) X-ray diffraction analysis.
Figure (2) Morphology of nitride layer by SEM micrographs for 10h (A) and 20h (B). CpTi layer at the green zone, heat-affected layer at the red zone and nitride layer at the blue zone.

Figure (3) Histomorphometric analysis for 2 weeks: (a) Microscopic photograph view of the non-treated implant after 2 weeks of implantation shows osteoid tissue with numerous bone cells around (arrow); (H&E stain) X20 and (b) High magnification view of implant treated with plasma nitride implant for two weeks duration shows new osteoid tissue, osteoblast cell (OB) and osteocyte cell (OC); H&E X20.

Figure (4) Histomorphometric analysis for 6 weeks: (a) Higher magnification view of the non-treated implant after 6 weeks of implantation shows new bone formation, osteocyte cell (OC) and osteoblast cell; H&E X20 and (b) Higher magnification view of bone trabeculae formation with implant treated with plasma nitride for the 6-week duration with a large number of osteocyte cell (OC); H&E X20.
References
