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ORIGINAL ARTICLE

Histomorphometric assessment of implant coated with mixture of nano-alumina and fluorapatite in rabbits

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KEYWORDS

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Abstract *Background:* The application of nanoscale surface modification was found to be useful in the improvement of osseointegration of endosseous dental implants. The fluorapatite (FA)/alumina (Al_2O_3) mixture is recognized for its outstanding bioinertia and can significantly increase the biocompatibility and bioactivity of biomaterials.

Objective: The aim of the present work was to evaluate the bone response to nano-alumina- and fluorapatite-coated dental implants using rabbit tibiae.

Material and Methods: The coating was performed using the dip-coating method. Commercially pure titanium screw-type implants were used as a control group. The coated implants were the experimental group. Each group consisted of 12 screws that were surgically implanted in 6 healthy New Zealand rabbits. Histological and histomorphometric evaluations were performed at the bone to implant contact (BIC) interface, bone fraction area occupancy (BAFO) and fibrous tissue at 2 and 6 weeks of healing.

Results: This analysis showed that the coated implants had more rapid osseointegration than the control group, with a significant difference after 2 and 6 weeks of healing for both groups. The histomorphometric evaluation demonstrated higher values for BIC% and BAFO% and lower values of fibrous tissue in the mixture-coated Ti implants than in the control group.

Conclusion: The current study suggested that the nano-alumina and fluorapatite mixture coating is a favourable candidate for rapid osseointegration over uncoated implants.

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1. Introduction

Implant surface topographical and chemical features play an essential role in osseointegration. An implant surface treatment can also be classified into physical, mechanical, and chemical methods to modify the surface topography and surface energy, resulting in enhanced wettability and increased

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cell proliferation and growth (Jemat et al., 2015). Bone-implant interface quality is directly influenced by implant surface roughness (Goyal and Kaur, 2012). The main methods that are reported to create implant roughness are acid etching, sandblasting, titanium plasma spraying and coating with bioactive material. A current trend is the manufacturing of implants with micro- and nanotopography to improve their biological characteristics (Rupp et al., 2018). Generally, fibroblasts and epithelial cells can attach firmly to smooth surfaces, whereas osteoblast cells prefer rough surfaces on which to attach, proliferate, and produce collagen (Bosco et al., 2012). Implants with moderate surface roughness also showed increased bone ingrowth and implant stability, allowing early mechanical loading (Schupbach, Glauser and Bauer, 2019).

Fluorapatite (FA) is a biocompatible and bioactive bioceramic material. It can release fluoride ions, which are osteoinductive and antibacterial (Uddin, 2018). FA has enhanced chemical stability, high interatomic bonding strength, improved thermal stability, low dissolution rate, and reduced bioresorption rate (Schupbach et al., 2019). FA can enhance bone apposition rates during the initial osteogenesis stage. These materials are also known for their lack of toxic and allergenic properties. The promising results of previous studies suggest that FA coatings may have many clinical advantages, leading to substantial interest in their use as dental implant coatings (Pajor et al., 2019).

One approach for increasing implant surface roughness is via the addition of Al_2O_3 using different methods. Al_2O_3 has highly suitable biocompatibility, fatigue and corrosion resistance, and mechanical strength. It is thermally stable, chemically inert, and has excellent wear resistance; hence, it is one of the preferred materials for orthopaedic applications (Li, 2017). Many studies have shown that the bonding strength of sintered HA/Al_2O_3 composite samples increases with higher Al_2O_3 content (Safarabadi et al., 2014). It appears that FA, added at 1 wt% to alumina, improves implant adhesion to bone (Ghorbel et al., 2016). The higher porosity of the coating due to the addition of FA to alumina can be helpful for bone ingrowth and aids mechanical fixation (Ghorbel et al., 2017). Adding FA to the alumina coating enhances implant adhesion on bone cells and ensures safe implantation (Ghorbel et al., 2019).

This investigation aimed to histologically evaluate the effect of coating commercially pure titanium implants with a nanofluorapatite-alumina mixture at the implant-to-bone interface in rabbit tibia.

2. Materials and methods

Twenty-four screw-shaped titanium implants (3.0 mm in diameter and 8 mm in length (the smooth portion was 3 mm, and the threaded portion was 5 mm)) were used. These implants were machined with a lathe cut machine from commercially pure titanium rods (grade II) (GRS Titanium Inc. 1550 Spruce Street, Wooster, Ohio 44691 USA). Acetone, alcohol, and deionized water were used for washing the screws in an ultrasonic bath, which were then left to dry at 45 °C.

The screws were divided into two groups: 12 screws left without coating as the control group and 12 screws coated with fluorapatite and alumina composite using the dip-coating method as the experimental group. FA powder was prepared by mixing $Ca_3(PO_4)_2$ with CaF_2 (Sigma-Aldrich, USA). The powders were milled and mixed at a molar ratio of 3:1 in ethanol for 48 h and left to dry. The mixed powders were condensed and then heated at 1000 °C for 3 h in air to produce FA powder (Ghorbel et al., 2019). The obtained FA powder was then mixed with 20% (wt.%) Al_2O_3 alumina (α- Al_2O_3) (Sky Spring Nanomaterials, USA) to obtain the dipping solution.

For the experimental group, the screws were placed into the dipping solution for 30 s and then removed and left to dry for 1 min at room temperature. The dip-coating process was performed electronically using a special dip-coating apparatus (HTDC-300 M, HTLAB, China) at a speed of 10 cm/min. This process was repeated to increase the coating thickness. Sintering the coated screws for densification was performed using a thermal treatment at 700 °C for 30 min in a Carbolite furnace. Inert gas (argon) was used during the treatment process to prevent oxidation of the coating, and all screws were stored in air-sealed sheets before gamma radiation sterilization (Kim et al., 2003).

Six healthy adult male New Zealand rabbits weighing approximately 2 kg were used in this study. This study was approved by the Animal Care and Use Committee at the College of Veterinary Medicine, University of Baghdad. Baghdad, Iraq. No. 1832 on 25 February 2018. They were left for 1 week in the same environments prior to surgical procedure and received standard food and water throughout the experimental period. To ensure that the animals were parasite free, a single dose of Ivermectin injection (10 mg) was given. After anaesthetizing the rabbits by intramuscular injection of ketamine HCl 25 mg/kg B. W (Rotexmedica, Germany) + Xylazine 17.5 mg/kg (Bayer Group, Germany) with Ketorolac (Rolac, Syria) 30 mg/kg B.W., the legs were shaved, washed, and decontaminated with ethanol.

With a sterile instrument, an incision line was made on the medial side of the tibia on both sides. A round drill was used for penetration to produce two holes with a 1 cm distance between them on each leg. The uncoated screw was then removed from the air-sealed plastic sheet and inserted into the initial hole (proximal one), and the second screw (coated with a combination of alumina and fluorapatite) was placed in the other hole (distal one). Three animals were sacrificed at two weeks, whereas the other three were sacrificed at six weeks post insertion of the screws. An en-block f the bone-implant undecalcified specimen of the screw with surrounding tissue was prepared by the sectioning of bone approximately 5 mm from the head of the screw using a disc cutter with slow rotating speed and cooling. Each specimen was fixed in 10% newly prepared neutral buffered formalin and left overnight.

Each bone-implant block was divided longitudinally into two parts with a cross-section using a sharp scalpel. The embedded blocks were sectioned perpendicularly to the longitudinal axis of the implanted screws. A total of 20 sections of 4 μm thickness were made for each block and for each healing time using a Leica SP1600 saw microtome (Leica, Nussloch, Germany). Staining of the tissue was achieved by placing the slide for 10 min in a container with haematoxylin and eosin stain (Dako, USA).

Immunohistochemistry staining was conducted to evaluate sclerostin expression (Abcam, optimal dilutions: 1/30) and alkaline phosphatase (ALP) (Abcam, optimal dilutions: 1/350). Osteocytes were labelled with sclerostin, and ALP were marked osteoblasts.

The histomorphometric assessment of the examined sections was carried out using a light microscope with a Samsung GT-N7100 camera, which was used to take photographs of each section. The histomorphometric parameters—bone-implant coating BIC, bone fraction area occupancy (BAFO) and fibrous tissues—were used to evaluate the response to the coating material.

The area of newly formed bone was identified according to standards determined by Shapiro (2008); this area appears similar to a rough meshwork (trabecular bone) of pinkish tissue that surrounds patches of lighter or pure tissue or matrix. ImageJ software (NIH Image, National Institutes of Health, Maryland, USA) was used to determine and measure the newly formed bone area.

The data obtained were analysed with IBM SPSS software (ver. 23, SPSS Inc, IL, USA) utilizing a Mann-Whitney *U* test each time. When p-values were < 0.05, differences were considered to be statistically significant.

3. Results

In general, all animals showed good postoperative healing with no remarkable side effects. Through the study period, they demonstrated normal movement without any signs of inflammation, infection, or allergic reaction at the surgical site. The interventional procedures had no effect on general health, behaviour, or feeding of the animals. The screws could not be moved by manual force.

The deposition of new bone for specimens obtained from the experimental group (i.e., the cpTi implants coated with a mixture of FA and Al_2O_3) was higher than for that obtained from the control group (i.e., the machined implant surface), both at two- and six-week intervals. Osteoblasts (osteoblastic osteoid) lining the new bone surface were found to be more prominent in the experimental group at a higher magnification. The histological findings after 2 weeks of implantation of uncoated implants showed a thread zone filled with woven bone, bone spicules, and numerous bone cells (Fig. 1A).

Histological views of the bone section next to the cpTi implants coated with a mixture of FA and Al_2O_3 2 weeks after the implant placement illustrated a thread zone filled with newly formed bone trabeculae; the area contained osteocytes surrounded by osteoblasts (Fig. 1 B).

Microscopic views of uncoated screws 6 weeks after implantation (Fig. 1 C) showed immature bone containing osteocyte cells arranged in an irregular manner in thick trabeculae and a considerable number of osteoblasts.

The microscopic findings for rabbit tibia bone sections around implants coated with an FA/Al_2O_3 mixture after 6 weeks of implantation revealed the active process of bone formation and substantial numbers of osteocyte cells compared to the control group (Fig. 1 D).

Immunohistochemical sclerostin staining was performed to assess changes in sclerostin expression. Sclerostin from the uncoated group showed the highest expression (Fig. 2). Immunohistochemical alkaline phosphate staining was performed to determine the effect of coating material on bone formation during healing. The coated group showed a significant increase compared to the control group ($p < 0.05$) (Fig. 3).

Quantitative histomorphometric analysis within the evaluation area revealed that the new bone area (%) was increased in both groups, but it was significantly more in specimens of the experimental group compared to those of the control group at both two- and six-week intervals. The mean BIC% of uncoated implants 2 weeks post implantation was 29.65 ± 10. 50; for coated implants after 2 weeks of implantation, this value was 40.52 ± 10.12% ($P < 00.05$). The BAFO% mean values were 47.32 ± 2.7% and 57.02 ± 5.3% ($P < 0.05$) for the uncoated and coated groups, respectively. At 6 weeks post implantation, there were statistically significant differences between groups: for BIC%, the mean values were 37.65 ± 8.6 and 54.6 ± 13.78 for the uncoated screws and coated commercially pure titanium implants, respectively ($P < 0.05$); the BAFO% mean values were 47.34 ± 4.9% for the uncoated group and 67.4 ± 5.9% for the coated group ($P < 0.05$). The mean values of fibrous tissue for the uncoated and coated groups at 2 weeks of implantation were 50 and 23 respectively. After 6 weeks of implantation, the mean value for the fibrous tissue of the uncoated group was 11, whereas this value for the coated group was 0.7 (Fig. 4).

Normality test showed normal distribution of data for all groups (for Shapiro test, p value greater than 0.05) except

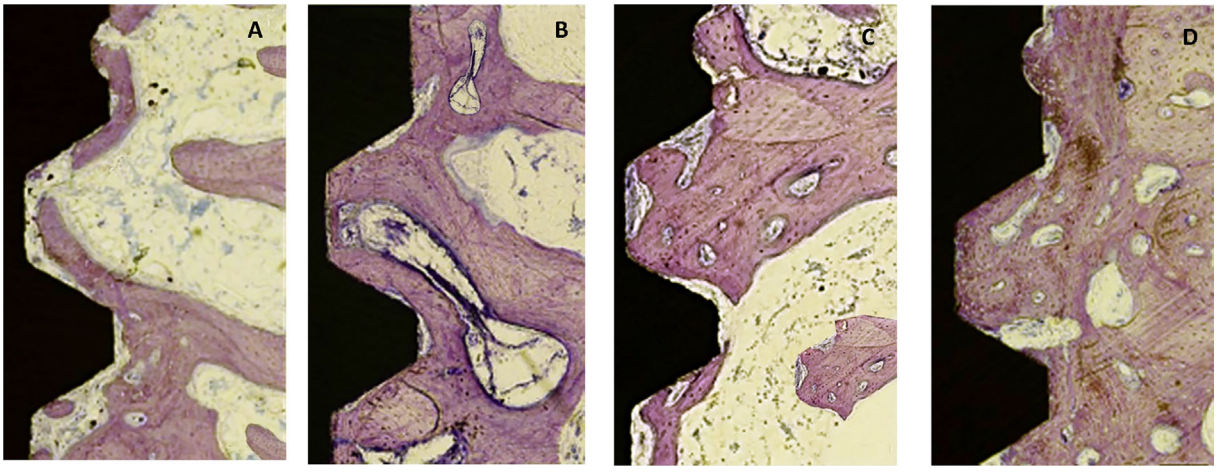

Fig. 1 Histological view of uncoated and coated screws (A:- uncoated screws after 2 weeks; B:- coated screws after 2 weeks; C:- uncoated screws after 6 weeks and D:- coated screws after 6 weeks).

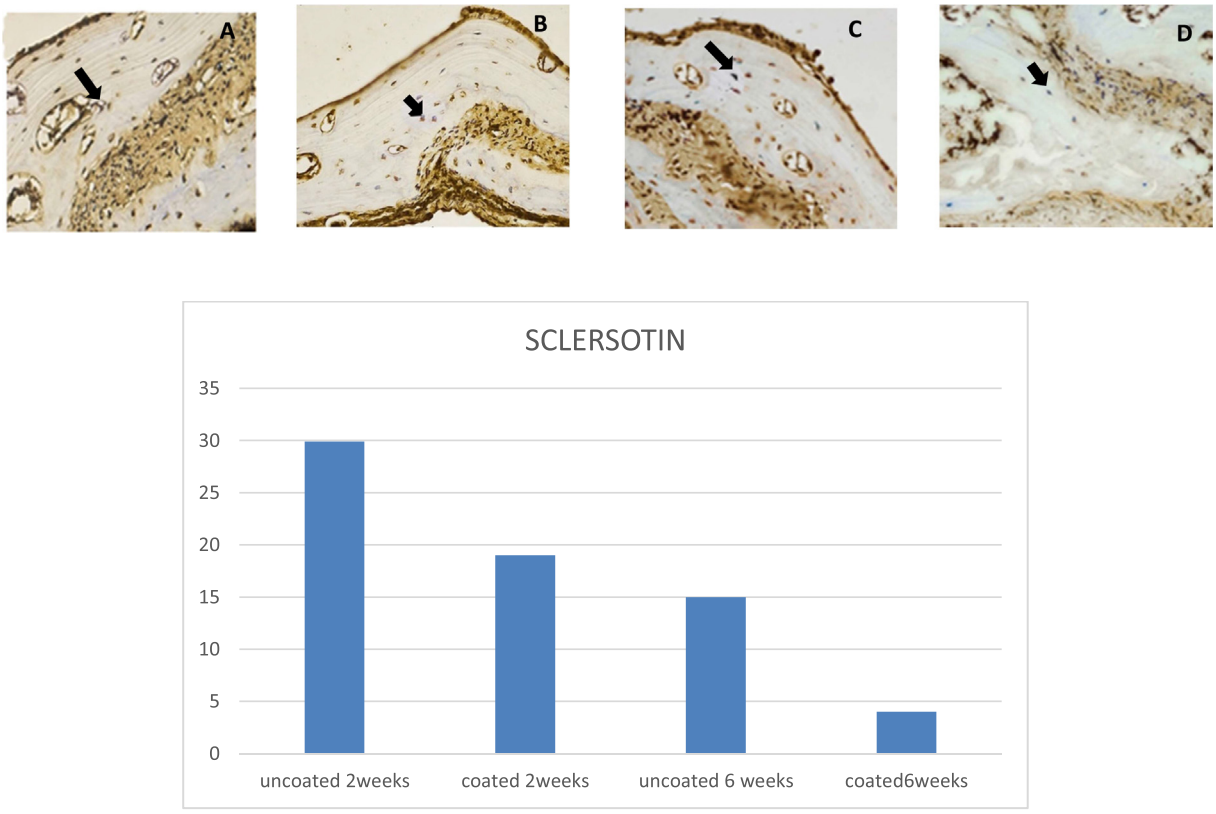

Fig. 2 Immunohistochemical sclerostin staining uncoated and coated screws (A:- uncoated screws after 2 weeks; B:- coated screws after 2 weeks; C:- uncoated screws after 6 weeks and D:- coated screws after 6 weeks).

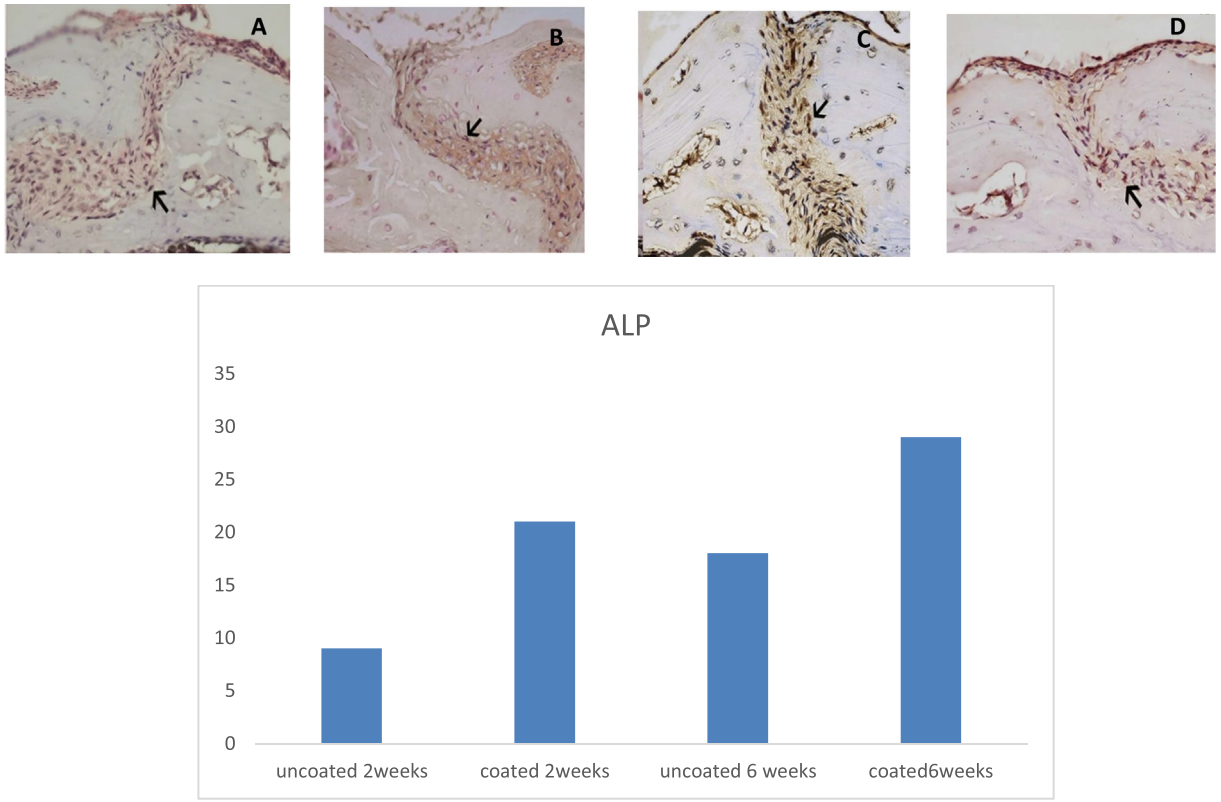

Fig. 3 Immunohistochemical alkaline phosphate staining uncoated and coated screws (A:- uncoated screws after 2 weeks; B:- coated screws after 2 weeks; C:- uncoated screws after 6 weeks and D:- coated screws after 6 week.

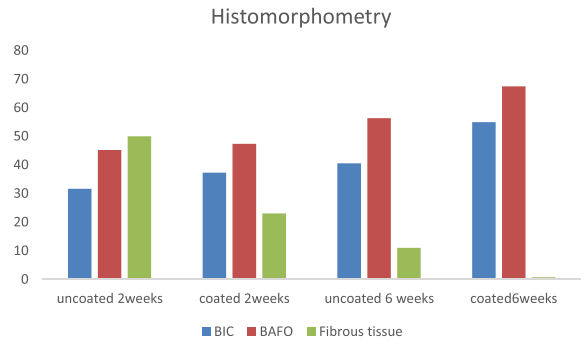

Fig. 4 Quantitative histomorphometric analysis.

for uncoated group at 2 weeks (for Shapiro test p value less than 0.05) (Fig. 5).

4. Discussion

This study analysed and compared the bone response to dental Ti implants with and without coating using a mixture of nano-alumina and fluorapatite. The evaluation was carried out via a histomorphometric assessment of the implant–bone interface during different healing times in rabbits. The current work's results demonstrated that coated Ti implants have a better bone response than commercially available uncoated Ti implants due to rapid osseointegration. The modification of implant surfaces is a relatively recent approach in dental implantology to promote osseointegration with bone. Despite the histomorphometric analysis being a destructive test, it is still considered to be a representative test for studying implant-to-tissue contact quality and evaluating implant stability. It can be applied at any time after the surgical insertion of the implant. This analytical approach has been used by many researchers to assess the bone-to-plant interface (Atsumi et al., 2007; Ghiban et al., 2006).

The histological examination of the coated group, two weeks after implantation, showed more evidence of new bone deposition surrounded by cells of osteoblasts and abundant osteocytes with active blood vessels at the thread–interface region compared to uncoated CpTi implants. At six weeks of healing interval, histological features for uncoated group specimens demonstrated that the implant–bone interface area was loaded with new bone, which was abundant, and there were osteocyte cells together with osteoblasts lining the Harversian canal. These findings indicate that the area of bone deposition was still being processed. On the other hand, the coated group specimens, six weeks post implantation, showed new dense bone that was almost mature, with osteocytes regularly arranged around the Harversian system. The present study showed that the placement of implants within living bone under an enhanced biological environment for bone regeneration, via the addition of promoting materials, resulted in better osseointegrated implants. This result agrees with Ibrahim et al. (2017).

The differences in the total amount of bone tissue formation around implanted screws in the cortical region and bone marrow were significant, indicating the high osteoconductivity of the coating surface.

Coated implants with a mixture of nano-alumina and fluorapatite showed higher mean values of new bone formation ratio when compared with uncoated screws. These findings may be due to the impact of FA (fluorapatite) on bone formation. Many researchers have found that the addition of FA to implants increased implant–bone adhesion (Jemat et al., 2015). This effect has been attributed to the chemical reaction of the compound. After implant insertion, the calcium and phosphorus ions "Ca and P" dissipate from the FA crystals, melt, and liberate Ca_2^+ and $(HPO_4)^{-2}$ around the implant. This process

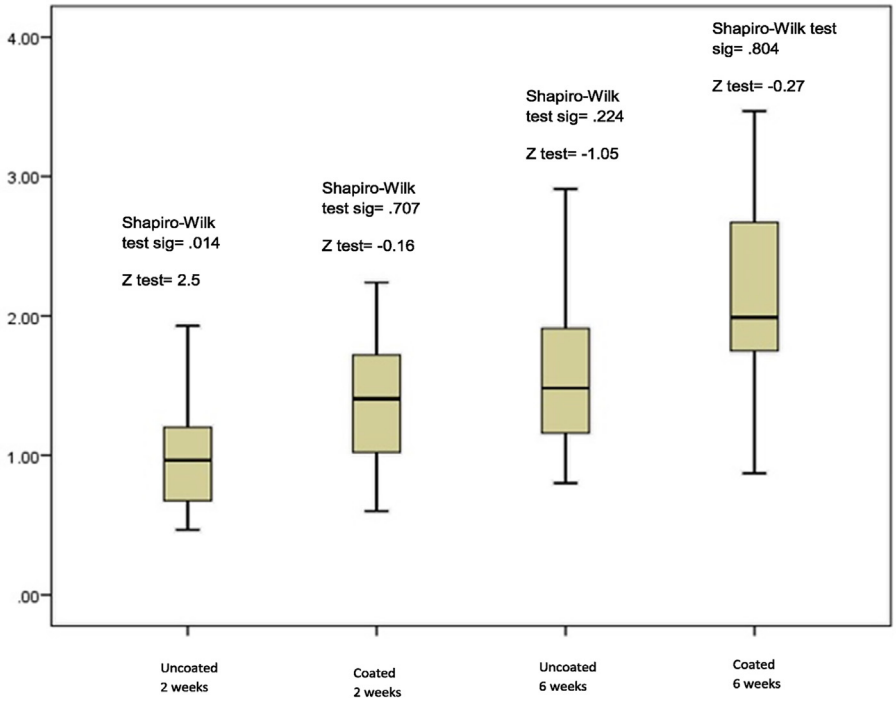

Fig. 5 Normality test.

increases the ionic strength and dispersion of the blood, leading to the deposition of apatite crystallites on the implant's surface. This sheet of apatite enables its proteins to promote adhesion of progenitor bone cells (Qiao et al., 2018), resulting in a more stable and bioactive structure (Dorozhkin, 2010). Fluoride-substituted HA (hydroxyapatite) within the bone structure can stimulate rapid bone formation and a more completely formed Haversian system in the mandibular jaw of dogs (Lobato, 2009).

Numerous kinds of fluoride-containing apatites have been shown to possess osteoinductive properties. These characteristics could be attributed to the existence of fluoride ions, which accelerate the proliferation and differentiation of osteoprogenitor cells and increase the number of osteoblasts that form bone at a faster rate, thus enhancing implant osseointegration (Pajor et al., 2019). Furthermore, FA has been shown to inhibit the maturation of osteoclasts and suppress phagocyte activity (Schupbach et al., 2019).

Although fluorapatite is biocompatible and has better stability, with fluoride released at a controlled rate that ensures the deposition of mechanically and functionally strong bone, the mechanical characteristics of fluorapatite and all other calcium phosphates are not suitable for many stress-carrying applications, as illustrated in *in vitro* studies (Ghorbel et al., 2019; Yoon et al., 2005). Fluorapatite bioceramics have a low density, resulting in weaker mechanical characteristics (Buzalaf and Whitford, 2011). Hence, many combinations of fluorapatite with other materials have been proposed to enhance these materials' mechanical properties (Schupbach et al., 2019; Gross and Rodríguez-Lorenzo, 2004). Alumina has excellent mechanical characteristics and increased affinity for fluorine, with which it can form a highly stable composite. Therefore, the alumina–FA compound is still considered to be the most suitable option for implants (Lee et al., 2007). Coating of cpTi implant surfaces using nano Al_2O_3 (20–100 nm) improved mesenchymal stem cell differentiation into osteoblast cells both in vitro and *in vivo*. The same observation was found when comparing Al_2O_3 coating implants with machined and acid-etched cpTi implants. In one study, the addition of Al_2O_3 to the implant showed increased fracture toughness and resistance to low-temperature ageing degradation (Igarashi et al., 2015). At the implant site, increased bone-specific gene expression in tissues adjacent to mixture-coated implants was observed (Mendonça et al., 2009).

It has been widely recognized that sclerostin inhibits bone formation because it prevents osteoblast differentiation (Ueda et al., 2016), and ALP is the characteristic marker of such differentiation (Ma et al., 2017).

The present work has some limitations. The number of studied samples was relatively small. The work was also accomplished with rather short healing phases. In addition, the research did not evaluate the implants' mechanical aspects under closed loading circumstances. Therefore, additional work is required involving prolonged healing periods and implant loading. Because of the preclinical nature of this study, the results should be extrapolated with caution to humans.

5. Conclusion

The present study evaluated the bone response of implants coated with a mixture of nano-alumina and fluorapatite in comparison to native commercial screws. Osseointegration was found to occur in both implant groups. The percentage values of coated implants were better than those in the control group, with more rapid bone formation and maturation in the experimental group at both periods of healing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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