Assessment of Calcium Carbonate Coating on Osseointegration of Commercially Pure Titanium Implant by Torque Removal Test and Histomorphometric Analysis

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Abstract

Background: One of the most important methods to replace lost teeth is dental implants. In order to increase the strength of connection of the implant with the jaw bone to provide early loading after placement, implant is coated by different coating materials that achieved that purpose. The aim of this study was to evaluate the influence of coating CP Ti implant with calcium carbonate on the strength of bone-implant interface after two and six weeks of implantation in rabbit femur bone by torque removal test, histological and histomorphometric analysis.

Materials and methods: Coating the surface of commercially pure titanium screws with extra pure synthetic calcium carbonate via electrophoretic deposition method (EPD) was done. The surface of disc samples after coating was checked by optical microscopy, X-ray diffraction examination and measurement of coating thickness. Ten male white French rabbits were prepared for implantation. Forty screws were implanted in the femur bone, two implant screws in each femur bone. The first screw is coated with calcium carbonate and compared with the second uncoated screw. Rabbits are divided into two groups according to the healing periods 2 and 6 weeks. By torque removal, the osseointegration is measured. Single screw from each group was used for histological and Histomorphometric analysis.

Results: There was significant increased mean torque removal for screws coated with calcium carbonate compared with uncoated screws. Histological examination showed an increase in the growth of bone cells for coated screws, and the histomorphometric analysis showed an increase in new bone formation percent (NBFP).

Conclusion: Coating the surface of the CP Ti implant with calcium carbonate via electrophoretic deposition method had great effect in increasing the osseointegration than uncoated surface.

Keywords: Calcium carbonate, commercially pure titanium, electrophoretic deposition method, Histomorphometric. **(J Bagh Coll Dentistry 2017; 29(1):32-38)**

INTRODUCTION

Nowadays and due to high rate of success, dental implant treatment becomes a well acceptable way for replacement missing teeth. The major factor for this success is the fact of osseointegration. (1)

Branemark in 1985 proposed that the osseointegration is "a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant".(2)

Osseointegration is influenced by the type of biomaterial used as implant, surface texture, type of machining, surgical procedure, bone quality and quantity and prosthesis design.(3)

Titanium as a biomaterial is used for construction the implants. Titanium is characterized by lightness and tolerance and has good chemical and mechanical properties makes it suitable for implant application. (4)

There is an interest in decreasing healing period following the implantation and the implant can be loaded safely by oral forces. Modification was done to the implant surface such as coating by different materials and by different techniques, and/ or modifying the technique of surgery in order to reduce the time of healing.(3)

One of the coating techniques is EPD. It is cheap method due to the simplicity of the equipment and the capability of depositioning many micro or nano materials and their combinations. (5)

Calcium carbonate ($CaCO_3$) is a restorable ceramic biomaterial that is gradually resorbed by the body.(6) Because of structural similarity with bone, corals as a source of $CaCO_3$ can be used for bone implants.(7)

Calcium carbonate has many medical uses, such as gastric anti-acid and nutritional calcium supplement. Also it works as a phosphate binder and used to treat the hyperphosphatemia. In

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pharmaceutical manufacturing, $CaCO_3$ can be utilized as inert filler for tablets.[8]

In this study, an extra pure synthetic $CaCO_3$ is deposited on CP Ti screws by electrophoretic deposition method (EPD) and implanted in rabbit femur and its effect on osseointegration is evaluated by torque removal test, histological and histomorphometric analysis after 2 and 6 weeks.

MATERIALS AND METHODS

Sample preparation

Grade 2 commercially pure titanium was used as a substrate for coating. By lathe machine, the titanium was cut into discs (2 mm thickness and 20 mm diameter). To have a uniform smooth surface, the discs were grinded using silicone carbide paper of 500 grit using a rotative grinding motion and polishing machine at 200 rpm for one minute.

The titanium discs were cleaned by solution of (3ml nitric acid, 1ml hydrofluoric acid and 6ml distal water). Then cleaning with ethanol alcohol using ultrasonic cleaner was done to eliminate any contamination and debris from the polished samples.[9]

Pilot study

A. Suspension preparation: the suspension was prepared by adding of $CaCO_3$ powder to the ethanol as a solvent (100 g/1 liter) in a glass beaker and adding iodine as a charging agent (2 g/L). By using a stirrer, the stirring was continued until a colloidal suspension was obtained. The suspension was maintained at room temperature for 48 hrs.

B. Electrophoretic deposition process: cathode electrode from power supply was connected to the CP Ti and anode electrode to a stainless steel plate. The distance between the electrodes was 1 cm. In order to select a proper voltage for the coating procedure, the power supply was used with different applied voltage (40, 50 and 60 V) for different time durations (0.25, 0.5, 1, 2 and 3 minutes.

C. Heat treatment: the coated specimens were densificated by sintering via tube furnace. Sintering is performed under argon gas (inert gas) in order to avoid oxidation of the specimen. $CaCO_3$ coated specimens were sintered to (400, 500 and 600) °C to select the proper heat temperature. Best results were obtained at 400°C for 1 hr. without loss of parts of coating and without cracks.

Examination of surfaces

A. Microscopical examination: the samples coating was examined by using optical microscope (MTI Corporation, USA) to examine the appearance of the coated surface of the sample. The micrographs were examined by a software in a computer.

B. X-Ray phase analysis: phase analysis was utilized on the coated disc samples using Shimadzu 6000- X-ray diffractometer using a Cu as target radiation at wave of 1.5406 A. Continuous scan with axis of 2ø angles were swept from 10- 70° in step of 0.05 degree.

Implant preparation:

Forty screws shaped implant were machined from the titanium bar using Lathe machine. The screw length was 8mm (3mm flat part and 5mm threaded part) and 3 mm in diameter. The height and width of the pitch is 1mm to fit the screwdriver during insertion and removal.[10] The screws were cleaned as mentioned in sample preparation.

The screws were divided into two groups, each group consisted of twenty screws. The first group of screws was the control, and the second group was coated with calcium carbonate for 0.5 min at 60 V as the same procedure of EPD that was performed on disc samples. The $CaCO_3$ coated screws were densificated by sintering to 400ºC for 1 hr. under argon gas. The screws were then sterilized with a physical mean of sterilization by gamma radiation (Figure 1).

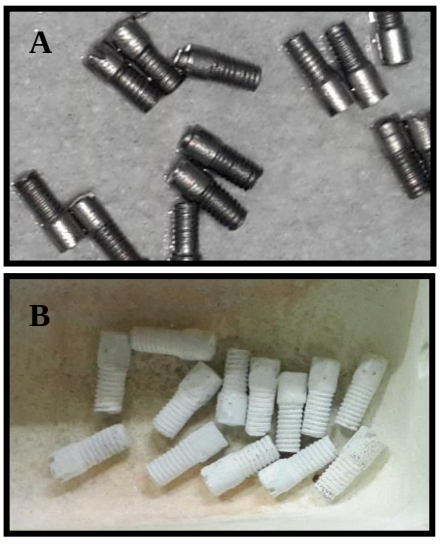

Figure 1: (A) Uncoated implant screws. (B) Coated implant screws with $CaCO_3$.

Animal and surgical procedures

Ten healthy adult male French rabbits weighing 1.5 - 1.75 kg (10-12 months of age) were used. Three days before operation, subcutaneous Ivermectin injection (0.2 ml) was given to eradicate the internal and external parasite. Intramuscular injection of an antibiotic (ceftriaxone) was given once daily (0.5ml) for 3 days to avoid any infection.

The rabbits were divided into two groups for 2 and 6 weeks healing periods, each group contained of five rabbits, one of them was killed for histological investigation using one leg and other leg for mechanical test, while the other four rabbits were used for mechanical test (torque removal test). Two screws were implanted in each femur of each rabbit (1 uncoated screw and 1 $CaCO_3$ coated screw for each femur bone).

General anesthesia was given to the animal by intramuscular injection of xylazine (0.7 ml/kg Body weight) and ketamine 10% (0.5 ml/kg Body weight). If the animal wake up during the operation, Isoflurane anesthetic inhalation was used (Isoflurane 1 bar with oxygen 1.5 bar).

The surgical instruments, gauze and towels were sterilized by autoclave at temperature of 134 C˚ at 2 bar for 3.5 minutes.

Both femurs were shaved using spray hair removal from outer side. Before placing the sterilized towel around the operation site, the skin was sterilized with alcohol and iodine. The incision was made on the lateral side, the skin and fascia was reflected, and blind dissection was made to the muscle to expose the distal side of the femur bone.

A round bur of 1.3 mm in diameter was used for bone penetration. Two holes with 1cm distance between them was made. The penetration was done by intermittent pressure at a rotary speed of 1500 rpm and reduction ratio of 16:1, and continuous irrigation with normal saline for cooling. Then the holes were enlarged gradually with fissure burs to 2.31 mm. $CaCO_3$ coated screw was implanted in the first upper hole via screwdriver until the screw was introduced completely into the bone. The uncoated screw was placed in the second hole.

Suturing of muscle's fascia was done with absorbable polydioxanone suture and the skin was sutured with silk suture. Rabbits then were followed for 2 and 6 weeks.

Mechanical testing (Torque test)

The same anesthetic solutions, instruments and materials were utilized as in the implantation procedure. The rabbits were anesthetized and incision was made on the lateral side and the skin, fascia and muscle were reflected to expose the implant. Torque measurement was performed by digital torque meter (TQ-8800, Taiwan) after supporting the femur bone to prevent any movement that may affect the test accuracy. After the screwdriver of the torque meter was engaged in the slit of the implant head, a torsional force was exerted for unscrewing the implant and the value was measured in Newton centimeters (N.cm).

Histological testing

One leg of one animal from each group of healing intervals was used for histological test. The animal was anesthetized with overdose of isoflurane general anesthesia.

The bone around the implant was cut by a disc cutter via prosthetic engine with straight hand piece (strong 90, Korea) with slow speed of rotation and normal saline irrigation. Bone-implant block was obtained by cutting about ½ cm away from the implant screw. The blocks were stored in 10% formalin for at least three days for fixation.

After preparation the slides, a light microscope (Pro.Way, China) was used and photographs of the sections were taken at 4, 10, 20 and 40 power magnification.

Histomorphometric analysis

New bone formation percent (NBFP) measurement was performed using Fiji ImageJ program (version 1.50b). First, the section diameter was measured and the mean value was inserted in the set scale box with the diameter of the screw. These values will be saved in the program as a data used to measure the area. Then the new bone areas were outlined and measured. The new bone formation percent (NBFP) was calculated according to the following formula: [11] [12]

$$\text{NBFP\%} = \frac{\text{Area of newly formed bone}}{\text{Total tissue area}} \times 100$$

RESULTS

X-ray diffraction of coated samples

The 2 ø angles were swept from 10- 70° in step of 0.05 degree. According to the (JCPDS), the

peak index was determined. International card for Diffraction Data (ICDD) PDF file # 44-1294 for titanium, # 11-0218 for Ti2O and # 29-0305 for $CaCO_3$.

After EPD (within 0.5 min. at 60V), as in **figure 2** the sample surface is seen completely coated with $CaCO_3$, because the diffraction peak was indexed to $CaCO_3$ phase matching the JCPDS file # 29-0305 for $CaCO_3$.

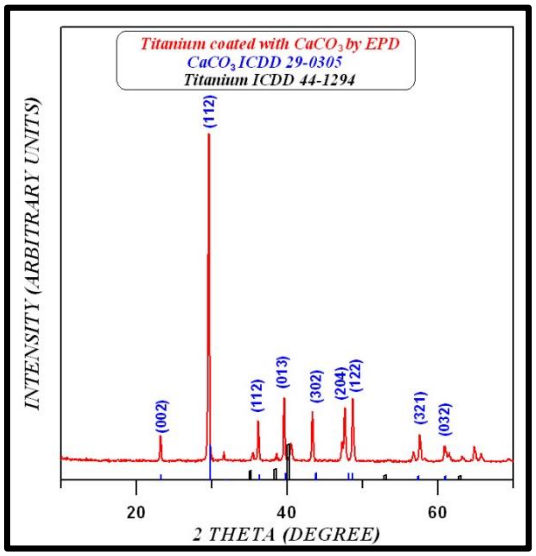

Figure 2: X-ray diffraction patterns of $CaCO_3$ coated CP Ti sample using EPD technique

Clinical observation

After healing period interval, at the time the animals are killed, the tissue surrounding the implant has negative clinical observation without any signs of severe infections. Stability of implants after each healing period was indicated by inability to remove the implant with manual force.

Mechanical testing

After 2 and 6 weeks of healing periods, $CaCO_3$ coated implants needed higher torque values to remove them, (mean value for 2 weeks: 4.6 N.cm & for 6 weeks: 12.7 N.cm), while uncoated implants needed less torque values (mean value for 2 weeks: 2.7 N.cm & for 6 weeks: 10.4 N.cm) (Table 1).

Table 1: Comparison of mean torque value of $CaCO_3$ coated and uncoated implants between both healing periods (N.cm)

Types	Time	N	Mean ± S.D.	Range
Control	2 wks	9	2.711 ± 0.853	1.5-4.0
	6 wks	9	10.477 ± 0.580	8.8-13.2
Coated	2 wks	9	4.688 ± 1.118	3.2-6.2
	6 wks	9	12.777 ± 1.504	9.8-14.6

t-test for equality of means of torque values between $CaCO_3$ coated and uncoated implants after 2 weeks of healing periods showed a highly significant difference at $p \leq 0.001$ and after 6 weeks of healing periods showed a significant difference at $p \leq 0.05$ (tables 2, 3).

Table 2: t-test for equality of means of torque value for $CaCO_3$ coated and uncoated implants after 2 weeks of healing period

Types	t	df	P-value	Sig.
Coated & uncoated	4.217	16	0.001	HS
HS: Highly significant at $p \leq 0.001$				

Table 3: t-test for equality of means of torque value for $CaCO_3$ coated and uncoated implants after 6 weeks of healing period

Types	t	df	P-value	Sig.
Coated & uncoated	3.162	16	0.006	S
S: Significant at $p \leq 0.05$				

t-test was also done to test the equality of means showed a highly significant differences at $p \leq 0.001$ between uncoated and coated groups at two period intervals (table 4).

Table 4: t-test for equality of means of torque removal value for uncoated and coated implants at 2 and 6 weeks intervals

Types	Time	t	df	P-value	Sig.
Uncoated	2x6 wks	12.973	16	0.000	HS
Coated	2x6 wks	12.942	16	0.000	HS
HS: Highly significant at $p \leq 0.001$					

Histological features

The histological feature of uncoated implants in the thread area after two weeks of implantation showed new bone trabeculae (BT) formation filled

the thread area. In addition, the histological feature showed active osteoblasts (OB) surrounding the periphery of BT, some of these cells are trapped in bone matrix as pre-osteocytes (POS) and then converted to osteocytes (OS). Also osteoclast are present (Figure 3). While histological findings of implants coated with $CaCO_3$ showed new bone formation surrounding the screw space. New bone trabeculae in the thread area are filled with new osteocytes (OS) and lined by osteoblast cells (OB). The reversal line separate between new and old bone. The new bone area shows woven bone (immature bone) filled with large number of osteocytes (Figure 4).

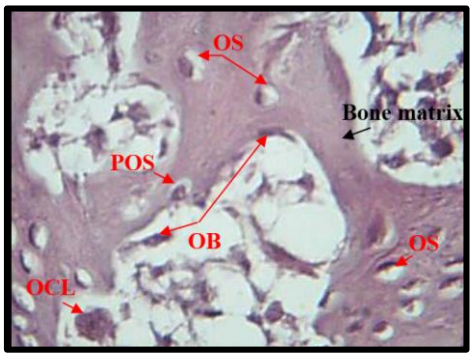

Figure 3: Microscopic view of uncoated implant after 2 weeks. Osteoblasts (OB), pre-osteocytes (POS), osteocytes (OS) & osteoclast (OCL). H & E ×40.

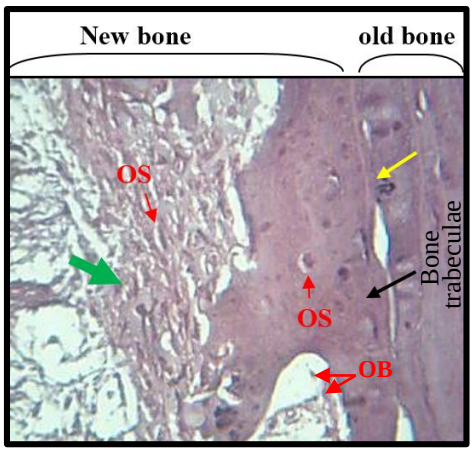

Figure 4: Microscopic view of $CaCO_3$ coated implant after 2 weeks shows new bone trabeculae in thread area filled with new osteocytes (OS) lined by osteoblast (OB). Reversal line (yellow arrow). Woven bone (green arrow) filled with osteocytes (OS). H & E ×40.

The thread area of uncoated implant after six weeks of implantation showed dense bone trabeculae (BT) filled with osteocytes (OS) surrounded by osteoblasts (OB) and osteoclasts (OCL) (Figure 5). While microscopic views for the coated implants with $CaCO_3$ shows active process of bone development, indicated by the active osteocytes arranged in circular pattern around Haversian canal (osteon formation) (Figure 6).

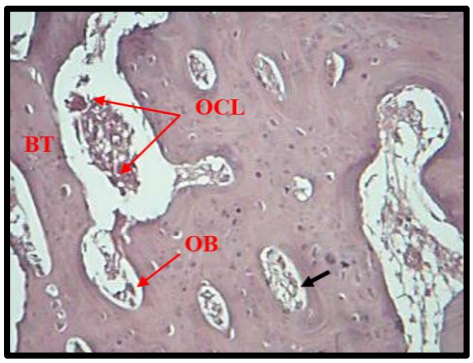

Figure 5: Microscopic view of uncoated implant after 6 weeks. Dense bone trabeculae (BT). Osteoblasts (OB). Osteoclasts (OCL). Haversian canal (black arrow). H & E ×20.

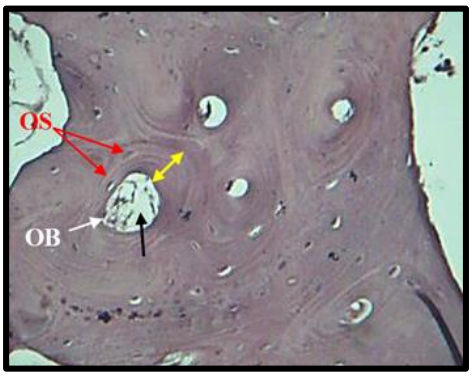

Figure 6: Microscopic view of coated implant after 6 weeks. Lamellae (Osteon formation) (yellow arrow). Haversian canal (black arrow). Osteoblasts (OB). Osteocytes (OS). H & E ×20.

Histomorphometric analysis

The new bone formation percent (NBFP) of the $CaCO_3$ coated implants in rabbit femur was greater than that of uncoated implants after 6 weeks of implantation. The mean of NBFP of $CaCO_3$ coated implants was 4.71 and for uncoated was 3.65 (Table 5).

Table 5: Descriptive analysis of NBFP of $CaCO_3$ coated and uncoated groups after 6 weeks of healing period

Types	N	Mean	S.D.	Range
Control	30	3.652	± 0.557	2.5-4.9
Coated	30	4.710	± 0.853	3.2-6.2

t-test for equality of means of NBFP values between $CaCO_3$ coated and uncoated implants after 6 weeks of healing period showed a highly significant difference (Table 6).

Table 6: t-test for equality of means of NBFP for $CaCO_3$ coated and uncoated implants after 6 weeks of healing period

Types	t	df	P-value	Sig.
Coated & uncoated	5.685	58	0.000	HS
HS: Highly significant at p≤0.001				

DISCUSSION

The purpose of surface implant treatment is to promote the osseointegration mechanism with stronger and faster bone formation, so better stability during the healing process is achieved permitting more rapid loading of the implant.(13)

Calcium carbonate is a biocompatible material that affects positively on the bone regeneration, osteogenesis and strengthening of the bone. It has been used as bone substitutes for high-speed bone resorption and for osteoconductive quality.(14)

Effect of calcium carbonate coating

Mechanical testing:

The $CaCO_3$ coated CP Ti screws placed in rabbit femur bone recorded a higher mean of removal torque value than uncoated screws after 2 and 6 weeks of implantation. This means $CaCO_3$ stimulated bone formation in which bond strength at the bone-implant interface was increased.

The positive role of calcium carbonate coating is dependent on calcium ions. As the $CaCO_3$ is a source of calcium, so increased amount of Ca may accelerate integrin-mediated attachment of bone-forming cells through enhanced ligand binding of receptor.(15) Ca has a positive effect on osseointegration by accelerating osteoblast proliferation, differentiation and adhesion after implantation.(16) In addition, carbonate phase of $CaCO_3$ is required for initiation of bone formation. Carbonate ions increase bioactivity and may be related directly to the process of dissolution-precipitation cycles that takes place during regeneration of bone tissue.(17) Carbonate ion substitutions have two types that occur in two atomic positions in the apatite lattice (type A & B). Type (A) substitution occurs when CO_3^{2-} substitutes for hydroxyl (OH^-) ions, and type (B) substitution occurs when CO_3^{2-} substitutes for phosphate (PO_4^{3-}) ions. (18)

Histological findings:

Histological features of uncoated CP Ti implant in femur bone after 2 weeks of implantation showed formation of primitive new bone near the surface of the implant. The new bone trabeculae were filled with active osteoblast cells which indicated the starting of bone formation. While the histological feature of coated implant showed many trabeculae of woven bone which were lined by bone forming cell (osteoblast) indicating active bone trabeculae formation. The bone trabeculae filled the thread region in the coated implant were thicker than that in the uncoated implant which indicate early bone stimulation.

Microscopical observation after 6 weeks of implantation revealed that the woven bone started to be replaced by lamellar bone to provide sufficient strength for load bearing. (19) In microscopical observation for uncoated implant, the thread still show immature bone filling (bone remodeling), while for coated implant showed more osteon formation (Haversian system) and beginning for the concentric arrangement of bone lamellae with their contained osteocytes, which indicate mature bone formation.

Histomorphometric analysis:

Histomorphometric measurement is an invasive method used to test the nature of the implant-tissue surface. It is used for several studies to evaluate the bone implant interface.(20)

Bone formation percent after 6 weeks of implantation was higher in $CaCO_3$ coated implant than uncoated implants. High bone formation percent after 6 weeks may be attributed to the activation of the $CaCO_3$ implant to the tissue at the interface at early stage and continuing of bone activation through the 6 weeks of implantation.

The growth and quality of newly formed bone tissue is affected by the surface properties of biomaterials that are necessary to the cells response at biomaterial interface.(21)

As conclusion; coating the surface of the CP Ti implant with calcium carbonate via electrophoretic deposition method had great effect in increasing the osseointegration than uncoated surface.

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الخلاصة

الخلفية: واحدة من أهم الطرق لتعويض الأسنان المفقودة هي زراعة الأسنان. ولأجل زيادة قوة التصاق الزرعة مع عظم الفك من أجل توفير التحميل الفوري بعد وضع الزرعات، تُطلى الزرعة بمختلف مواد الطلاء لتحقيق ذلك الغرض. إنّ الهدف من هذه الدراسة هو تقييم تأثير طلاء سطح زرعة التيتانيوم التجاري النقي بمادة كاربونات الكالسيوم ومدى قدرتها على زيادة قوة التصاق الزرعة بالعظم بعد أسبوعين وستة أسابيع من زراعتها في عظم فخذ الأرنب بواسطة قياس عزم التدوير والتحليل النسيجي.

المواد وطرق البحث: طلاء سطح براغي التيتانيوم التجاري النقي بمادة كاربونات الكالسيوم النقي الصناعي بطريقة الهجرة الكهربائية. تم فحص العينات المطلية والتأكد من طلائها بشكل موحّد عن طريق الفحص المجهري الضوئي وفحص حيود الأشعة السينية وقياس سمك الطلاء. عشرة أرانب ذكور فرنسيات بيضاء اللون تم تحضيرها لزراعة 40 برغي في عظم الفخذ، برغيان في كل عظم. البرغي الأول مطلي بكاربونات الكالسيوم ويقارن مع البرغي الثاني الخالي من الطلاء. تقسم الأرانب إلى مجموعتين حسب مدة الشفاء (اسبوعان وستة أسابيع). بواسطة فحص عزم التدوير تُقاس قوة التصاق البرغي بالعظم، ويُترك برغي واحد من كل مجموعة للفحص والتحليل النسيجي.

النتائج: اظهرت النتائج زيادة معدل عزم التدوير للبراغي المطلية بكاربونات الكالسيوم النقي الصناعي مقارنة مع البراغي غير المطلية. وأظهر الفحص النسيجي زيادة في نمو الخلايا العظمية في البراغي المطلية، أمّا التحليل النسيجي فقد أظهر زيادة نسبة معدل النمو العظمي.

الاستنتاج: طلاء سطح الزرعة بكاربونات الكالسيوم بطريقة الهجرة الكهربائية كان له تأثير كبير في زيادة ارتباط الزرعة بالعظم مقارنة مع زرعة غير مطلية.

الكلمات الرئيسية: كاربونات الكالسيوم، التيتانيوم التجاري النقي، طريقة الهجري الكهربائية، التحليل النسيجي.