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Localization of Osteocalcin in Amelogenin-Propolis Coated Dental Implants in Rabbits

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Abstract
Dental implant is an artificial tooth root fixed into the jaws to hold a replacement tooth or bridge. Functional surface modifications by organic material such as amelogenin/propolis coating seem to enhance early peri-implant bone formation. The aim of the study was to evaluate the expression of osteocalcin as bone formation markers in amelogenin/propolis coated and uncoated implant in interval periods(1,2 and 4 weeks). Commercially pure Titanium(cpTi) implants, coated with amelogenin/ propolis, were placed in the tibias of 30 New Zealand white rabbits, histological and immunohistochemical tests for detection of expression of osteocalcin were performed on all the implants of both control and experimental groups for (1,2 and 4 weeks) healing intervals. Histological finding for coated titanium implant with amelogenin/ propolis illustrated an early bone formation, mineralization and maturation in comparison to control. Immunohistochemical finding showed that positive reaction for osteocalcin was expressed by osteoblast cells (OB)at implants coated with amelogenin/ propolis, indicating that bone formation &maturation was accelerated by adding biological materials as a modification modality of implant surface. The present study concludes that coating of implants with amelogenin/ propolis showed increment in osseointegration in short interval period.
Keywords: amelogenin, propolis, dental implant, biochemical bone markers, osteocalcin and osseointegration.

1. Introduction

Dental implant is an artificial tooth root fixed into the jaws to hold a replacement tooth or bridge(Alghamdi *etal*. 2013). Titanium is widely used for dental implants because of its biocompatibility, mechanical strength and plasticity for prosthetic design. Osseointegration refers to the growth of bone as it incorporates surgically implanted materials(Bougas *etal*. 2012). In order to enhance bone formation, implants have been coated with bone specific biomolecules(Geng-Sheng etal. 2009). Many kinds of bioactive materials used to coat the surfaces of dental implants(Oida *etal*. 2002). Amelogenins is the major organic component in the enamel matrix of developing teeth and plays an important role in enamel biomineralization(Haze *etal*. 2007). Amelogenins are hydrophobic enamel proteins secreted by ectodermal cells – ameloblasts – during enamel. Osteoblasts, odontoblasts and bone marrow stromal cells also express the amelogenin(Veis *etal*. 2000). Propolis is the most important chemical weapon of bees against pathogenic microorganisms. It is a sticky, resinous substance collected by honey bees from the sap, leaves, and buds of plants, and then mixed with secreted beeswax(Hellner *etal*. 2008). Study by Chai *etal*. 2005, investigated that the attenuation of osteoclastogenesis & induction of osteoclast apoptosis through the inhibition of nuclear factor-kB (key regulator of osteoclast differentiation, activation & survival activation by the propolis caffeic acid phenethyl ester, this might be useful for the treatment of osteolysis attended with enhanced osteoclast formation & activation. Sabir *etal.*2005, reported the Propolis is capable of stimulating the production of (TGF)-beta1. Osteocalcin, the γ-carboxyglutamic acid-containing protein, which in most species is the predominant noncollagenous protein of bone and dentin, has been postulated to play roles in bone formation and remodeling(AL-Zubaydi *etal*. 2011). Osteocalcin is secreted solely by osteoblasts and is pro-osteoblastic, or bone building, by nature. It is also implicated in bone mineralization and calcium ion homeostasis(Al-Ghani *etal*. 2011).

2.Material and Methods

Sixty machined surface Iraqi implants from commercially pure titanium rod were inserted in 30 male adult white New Zealand rabbits. TwoTitanium implants were placed in the tibia of each rabbit. The animals were scarified at 1 , 2 and 4 weeks after implantation (10 rabbits for each interval). Animals were generally anaesthetized and atruamatic surgical technique was performed to prepare two holes in the tibia, amelogenin / propolis(AP) coated implant was inserted in one hole and uncoated implant (control) placed in the second one.

All tissue specimens, samples and controls, were fixed in 10% neutral formalin and processed in a routine paraffin blocks. Each formalin-fixed paraffin-embedded specimen had serial sections were prepared as follows: 4µm thickness sections were mounted on clean glass slides for routine H&E staining procedure from each block of all studied sample. Other 4 sections of 4µm thickness were mounted on positively charged microscopic slides for immunohistochemical localization of osteocalcin. The procedure of the IHC assay was carried out in accordance with the manufacturer instructions of Anti-Osteocalcin antibody (ab13418) Abcam UK and

Detection Kits System **(**ab 94740) Abcam UK.

3.Results

3.1 Histological examination

One week postoperatively, the AP-coated implant showed bone trabeculae filled thread area. Osteoblasts arranged on the periphery of these trabeculae and osteocytes were embedded within it(figure1).On the other hand, large number of fatty cells and blood vessels filled threads of uncoated implant (figure 2).

The histological picture of 2 weeks AP-coated implant illustrates dense bone thread that almost filled the entire threads of the implant with numerous osteocytes and few osteoblasts (figure 3). While the uncoated implants shows a number of active osteoblast and progenitor cells scattered within woven bone, with few thin bone trbeculea involve with preostecytes and ostecytes (figure 4).

Regarding 4 weeks postoperatively, the AP- coated implant reveled mature bone thread with haversian system (figure5).The histological view of uncoated implants showed thin bone trabeculea filled implant threads(figure 6).

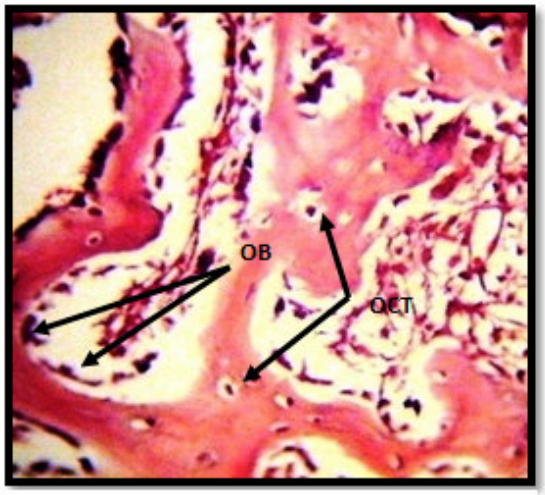

Figure1: View of AP-coated implant in 1 week interval Shows bone trabeculae osteoblast(OB), osteocytes (OCT), H&E X40

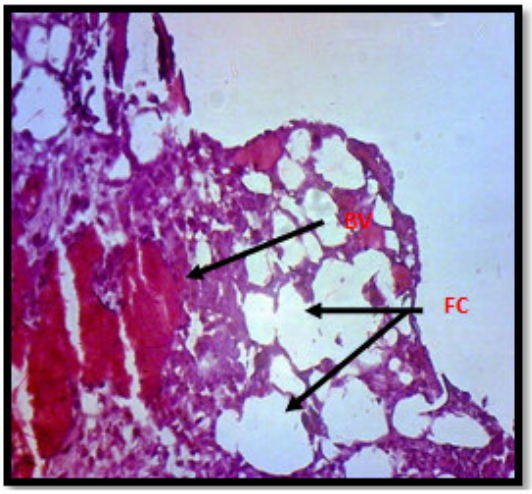

Figure2 : View of uncoated implant in 1 week interval, shows thread filled with large fat cell(FC), blood vessel(BV). H&E X20.

Figure 3: View of 2 weeks thread in AP-coated implant show dense bone thread show bone trabeculae(BT) , osteoblast(OB) and osteocyte (OCT), H&E X40

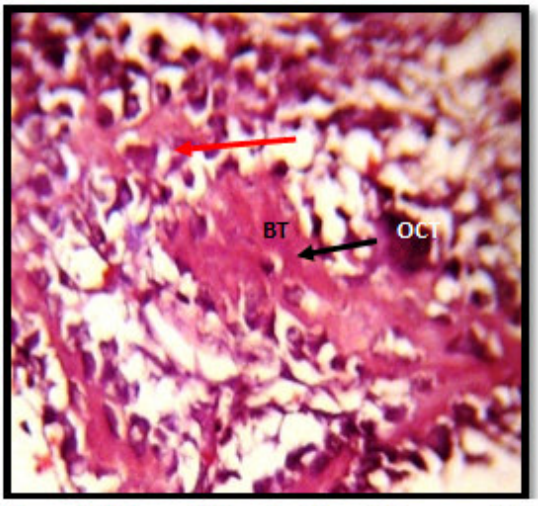

Figure4: View of 2 weeks thread in uncoated implant show thin bone trabeculae(BT) , active osteoblast(OB), preosteocyte(red arrow) and osteocyte (OCT). H&E X40

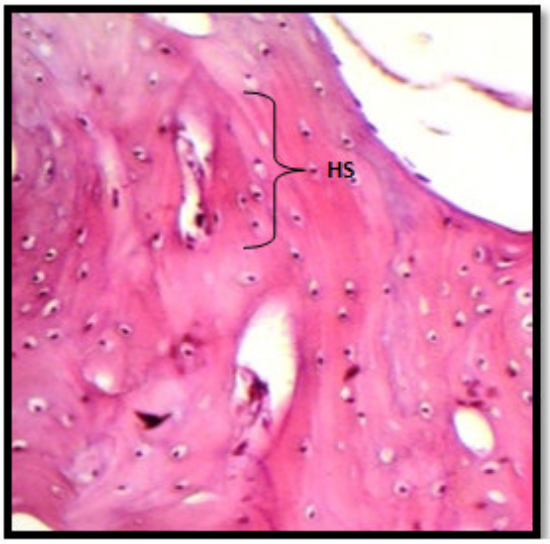

Figure5: View of 4 weeks thread in AP-coated implant show mature bone thread with haversian system(HS).H&E X40.

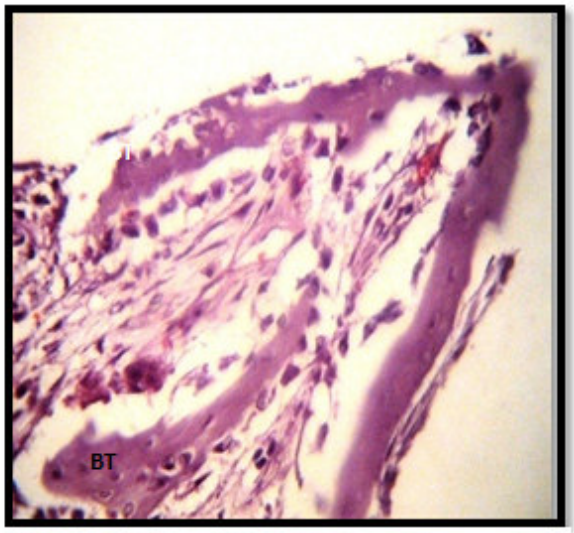

Figure6: View of 4 weeks thread in uncoated implant show thin bone trabeculae(BT) , H&E X40

3.2 IHC examination for osteocalcin (OC)

The immunohistochemical staining with OC monoclonal antibody of 1 week AP-coated implants showed moderate positive expression in the osteoblasts, progenitor, steocytes cells and in extracellular matrix (figure7). While the uncoated implant showed negative expression of OC in progenitor and extracellular matrix in thread area (figure8). On the other hand, the OC expression was strong in osteoblasts, osteocytes and extracellular matrix of 2 weeks AP-coated implant (figure 9). The uncoated implant showed that OC expression was negative in the threads of the same interval (figure 10).

Furthermore, the localization of OC expression was negative in osteoblasts and osteocytes of 4 weeks interval of AP- coated implant (figure11). While uncoated implant showed moderate positive expression in osteoblasts, progenitor cell and in extracellular matrix (figure12).

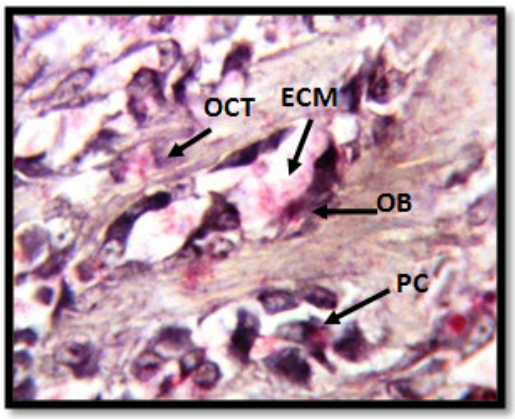

Figure7: View for positive IHCred stain for localization of OC of AP-coated implant for 1week interval, in osteoblasts (OB), osteocytes(OCT), progenitor cells (PC) and extracellular matrix (ECM), Fast red stain with counter stain hematoxylin, X100.

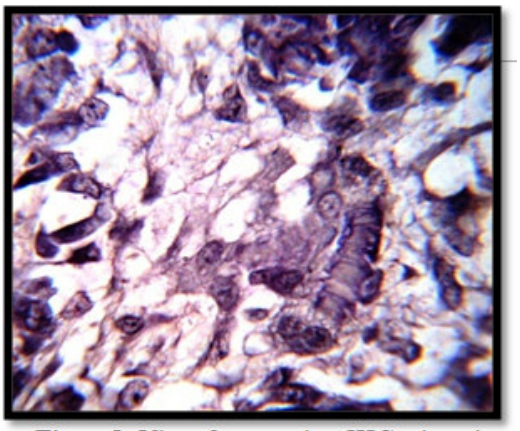

Figure8: View for negative IHCred stain for localization of OC of uncoated implant for 1week interval, Fast red stain with counter stain hematoxylin, X100

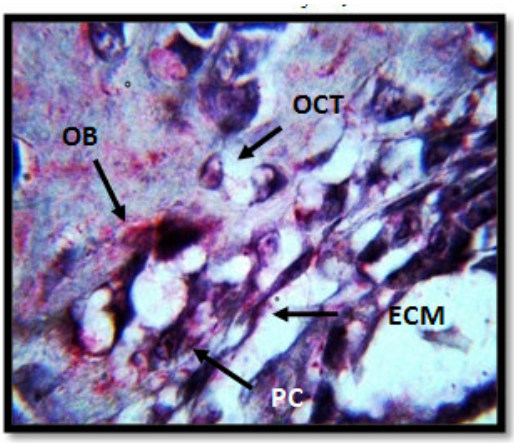

Figure9: View for positive IHCred stain for localization of OC of AP-coated implant for 2weeks interval, in osteoblasts (OB), osteocytes(OCT), progenitor cells (PC) and extracellular matrix (ECM), Fast red stain with counter stain hematoxylin, X100.

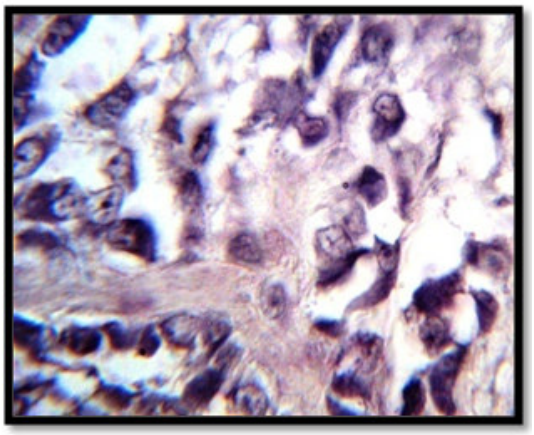

Figure10: View for negative IHCred stain for localization of OC of uncoated implant for 2weeks interval, Fast red stain with counter stain hematoxylin, X100

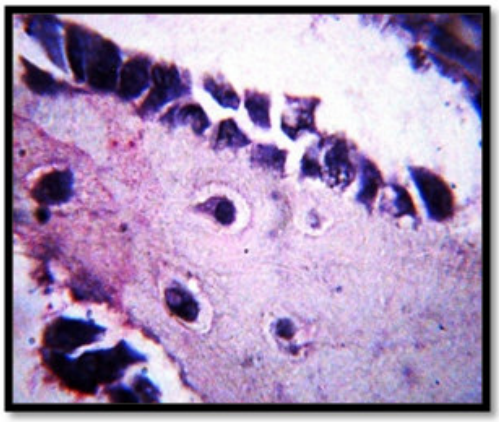

Figure11: View for negative IHCred stain for localization of OC of AP-coated implant for 4weeks interval, Fast red stain with counter stain hematoxylin, X100

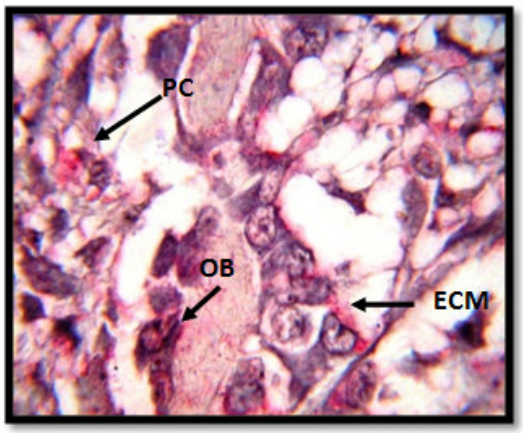

Figure12: View for positive IHCred stain for localization of OC of uncoated implant of 4weeks interval in osteoblast(OB), progenitor cells (PC) and extracellular matrix (ECM), Fast red stain with counter stain hematoxylin, X100.

4. Discussion

According to our knowledge there was no previous study concerning the combination of amelogenin and propolis coated implant, so this study regard as the first one. The results of this combination illustrate enhancement of bone formation around titanium implants by the formation of bone trabeculae from the first week interval and formation of mature bone in four weeks. This result agree with Tanimoto *et al*., 2012, who found that amelogenins enhances the mineralization accompanied by the upregulation of bone markers in human bone marrow MSCs during osteogenic differentiation, suggesting a certain role of amelogenin in the modulation of osteogenic differentiation of MSCs. Al-Molla, 2007, showed that propolis increase the bone formation in one week in comparing to the control group. Also this study agree with **Altan *et al.*, 2013, who found that the** use of propolis may hasten new bone formation at the expanded suture in rats after 12 days of mechanical retention.

The amelogenin and propolis enhance mesenchymal stromal cell to differentiate to preosteogenic that secret OC and expressed by osteoblast. This study illustrate positive OC expression in active mitotic osteoblast, and progenitor cells in all Titanium-coated groups and negative in uncoated group at 1 week interval. This expression increased within 2 weeks after implantation and then decrease with time. This result agree with Novaes *et al.,* 2010, who reported that osteocalcin, as one of the important indicators of osteogenic differentiation and bone tissue formation, have been shown to express at higher levels on modified titanium surfaces.

Regarding negative expression of uncoated titanium implant after 2 weeks postoperatively was agree with study by Trombelli *et al.* 2008, who used the antibodies against osteocalcin, for osteoblast and osteocyte cells. In

control group, the density of positive cells increased from 4 weeks to 6–8 weeks.
So finally, mixing of bioinert propolis with a biological material (amelogenin protein), proved to increase the bioactivity of the product and to promote mechanical properties of the implant and enhanced the osseointegration during healing period. Osteocalcin marker regard as an important indicators of osteogenic differentiation and bone tissue formation, have been shown to express at higher levels on modified titanium surfaces especially in early healing periods(Novaes *et al.,* 2010).

Conclusion

Mixing of bioinert propolis with a biological material (amelogenin protein), proved to increase the bioactivity of the product and to promote mechanical properties of the implant and enhanced the osseointegration during healing period. Osteocalcin marker regard as an important indicators of osteogenic differentiation and bone tissue formation, have been shown to express at higher levels on modified titanium surfaces especially in early healing periods.
Suggested clinical application of amelogenin and propolis coating material for enhancing the osseointegration around the dental implant and in tooth extracted sockets in order to minimize symptoms associated with the healing process.

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