In vivo study of the effect of collagen protein coated implant as compared with implants coated with a mixture of partially stabilized zirconia and collagen on osseointegration

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ABSTRACT
Background: Biocompatibility of orthopaedic surgical implants with bone tissue allows adequate osseointegration between the bone and implant. To achieve this, implants are coated with biocompatible materials. Commercially pure titanium is widely used as dental implant material because of its suitable mechanical properties and excellent biocompatibility, and to enhance osseointegration of the implant, organic and inorganic materials are used as a bioinert coating material.

Aim of the study: Comparison of the influence of the implant coated by biological material (collagen), and implant coated by both bioinert ceramic (zirconia) and collagen, on osseointegration by immunohistochemical, and radiographical studies with mechanical test.

Materials and Methods: Commercially pure Titanium (cpTi) implants, coated with PSZ powder by electrophoretic deposition (EPD) method, collagen protein, and mixture of collagen and partially stabilized zirconia (PSZ) were placed in the tibias of 12 New Zealand white rabbits. Immunohistochemical tests for detection of expression of osteocalcin and growth hormone receptor were performed on all the implants of both control and experimental groups for (3 days, 1, 2, and 6 weeks) healing intervals. Mechanical test (torque removal test) was performed as an indicator for the presence of osseointegration and as a test for the mechanical property of bone-implant interface.

Results: have illustrated that removal torque mean values, in all studied groups (uncoated and coated with both PSZ and collagen at (2 & 6 weeks) healing intervals were increasing with advancing time (higher at 6 than other periods), also results have shown that positive reaction for osteocalcin (OC) & growth hormone receptor (GHR) was expressed by osteoblast cells (OB) at implants coated with collagen and implants coated with zirconia and collagen, indicating that bone formation & maturation was accelerated by adding biological materials as a modification modality of implant surface.

Conclusion: It is concluded that coating of implant with collagen and coating with both PSZ and collagen showed an increment in osseointegration in short interval period.

Key words: dental implant, collagen, zirconia, biochemical, electrophoretic deposition, bone markers, osteocalcin, growth hormone receptor.

INTRODUCTION

Bone, as a structural tissue, has the ability to repair itself by a process called “bone remodeling” which consists of older bone tissue (resorption) and replacement with new bone tissue (formation) (1). Bone formation markers indicate osteoblast activity (2). Osteocalcin is used as a clinical marker for bone turnover in physiological and pathological conditions (3). Growth hormone (GH) is able to effect many cellular processes other than growth. GH is able to exert its pleiotropic effects through the GH receptor (GHR) which is a transmembrane receptor for growth hormone (4), it can stimulate many diverse signaling pathways leading to different cellular responses (5).

Functional implant surface modifications such as collagen coating seem to enhance early peri-implant bone formation (6). It has been reported that osteoblast activity was significantly increased around loaded Ti/ Coll screws compared to uncoated ones (7). In vitro evaluation of human mesenchymal cells have shown, enhanced cell growth on collagen coated surfaces as compared to uncoated pure titanium, and that integration in trabecular, or cancellous, bone can be enhanced by the surface collagen layer (8). Zirconia (zirconium dioxide, ZrO2) is a bio-inert non-resorbable metal oxide that improves cell proliferation significantly during the first days of culture, but it does not improve attachment and adhesion strength. Zirconium oxide may therefore be a suitable material for dental implants (9). Electrophoretic deposition (EPD) can be applied to a great variety of materials to produce uniform deposits (coatings) with high microstructural homogeneity, to provide adequate control of coating thickness and to deposit thin and thick
films on substrates of different shapes (10). Our study was designed to evaluate the effect of using collagen and using of mixtenure of PSZ and collagen as a coating of dental implant.

MATERIALS
-CpTi readymade implants from Friatec AG company were modified and machined in diameter about 3.5 mm, length of 8mm (5mm was threaded and 3mm was flat).
-Collagen protein (No.C-7521, SIGMAp).
- Partialy Stabelized Zirconia powder (ZIRCONIA SALES-GU 185 SS-U.K.).
- Phosphate ester (Emphos PS-21A, Witco).
- Ethanol 99.8% (GFS Chemicals, Germany).
- Osteocalcin (OC) (OC4-30, AB13419), & growth hormone receptor (GHR) (MAB263, abcam/U.K., 11380) monoclonal antibodies were used in the present study.
- Ab anti Mouse HRP/DAB detection kit (abcam/U.K., 6425-15):
- Hydrogen peroxide Block.
- Protein Block.
- Biotinylated goat anti-mouse IgG.
- Streptavidin peroxidase.
- DAB chromogen

METHODS
Thirty-six commercially pure titanium implants (cpTi) were used in this study, group I includes (12) implants were coated with PSZ by electrophoretic deposition (EPD) method. EPD suspension was prepared by adding PSZ fine powder to the solvent which was ethyl alcohol (100gm/1 litter. Phosphate ester (3.5g/1 liter) was added to the suspension as a dispersant agent, followed by adding the binder (3g/1liter) of poly vinyl butyral. a colloidal suspension obtained (11), and then mixed with collagen, group II (12) implants coated by collagen only. (collagen was added immediately after implantation), the rest (12) implants, group III were used as controls.

Surgical procedure:
Animals were generally anasthesized and atraumatic surgery was performed to prepare suitable size holes (3.0mm), in the tibia to place the implants, X-ray was taken immediately after the surgery to ensure that the implants were properly inserted in their positions, scarification of the animals was done after 3 days, 1, 2, and 6 weeks.

RESULTS
Clinically all implants at the day of sacrifice were found stable in the bone. They could not be moved with manual force and there were no detectable peri-implant defects at the coronal aspect of any implant for all healing intervals (3 days, 1, 2 and 6 weeks). Radiographic evaluation have shown that there were no areas of radiolucency between the implant and adjacent cortical bone in any specimen of radiographic examination and there was no gross change in the tibial architecture noted in all specimens for the six weeks healing period as shown in figures 1, and 2, where (C) the control, and the collagen coated (COLL) & collagen & PSZ coated by EPD (ZEC) are the experimental implants.

Figure 1: Conventional radiographic view for implant coated with collagen for 6 weeks shows cortical bone thickness and radiopacity surrounded the implant.

Figure 2: Conventional radiographic view for Ti implant coated zirconia & collagen (ZEC) for 6 weeks shows overhang bone on the implant.

Mechanical testing:
The equality of variance and the equality of means between all groups of implant in each healing interval tested by Levene's test and ANOVA (Table 1) revealed that there is a highly significant difference at p≤0.000 in torque value between different groups (including uncoated and coated with collagen, and coated with zirconia by EPD and collagen, at 2 and 6 weeks healing intervals.

Immuno-histochemical examination for osteocalcin (OC) expression of implant in 3 days, 1, 2, and 6 weeks intervals periods:
Three days after implantation:
A- Uncoated CpTi implants: the immuno-histological view illustrates primitive new bone formation in which future bone is formed as
embryonic type. This type of bone is characterized by presences of progenitor cells that are scattered randomly, which show positive expression of osteocalcin protein (Figure 3).

B- CpTi implants coated with collagen: Histological findings of the implant coated with collagen after 3 days duration shows bone marrow with stroma cells as a large number of active progenitor cells, associated with presence of fat cells (Figure 4).

C- CpTi implants coated with zirconia & collagen (ZEC): Titanium implant coated with zirconia and collagen protein surrounded by marrow bone with the presence of neighbouring fat cells, and many progenitor cells are present nearby which show positive reaction for OC (Figure 5).

One week after implantation

A- Uncoated CpTi implants: Embryonic connective tissue with active collagen fiber deposition which represent organic constituents of bone, also active fiber forming fibroblasts are seen, positive DAB staining is indicated by the yellowish brown color (Figure 6).

B- CpTi implants coated with collagen: Woven bone formation and some areas of osteoid tissue, both stained positively, blue colored basal bone is seen which illustrates negative stain (Figure 7).

C- CpTi implants coated with Zirconia & collagen (ZEC): Microphotograph view of immunohistochemical localization of OC in rabbit tibia at implant coated with zirconia (EPD) and collagen protein illustrates woven bone formation with positively stained osteoblast cell nuclei within extracellular matrix (Figure 8).
Two weeks after implantation

A- Uncoated CpTi implants
View of uncoated Ti implant in rabbit tibia after two weeks of implantation shows a number of active osteoblasts scattered within woven bone, they show positive DAB stain for OC(Figure 9).

B- CpTi implants coated with collagen
Microphotograph view of bone section related to collagen coated implant after 2weeks of implantation, shows numerous anastomosing trabeculae. These trabeculae surround primitive marrow cavities of different sizes. Osteocytes are embedded in the bone trabeculae and osteoblast cells are seen lining them (Figure 10).

C- CpTi implants coated with zirconia & collagen (ZEC):
The histological view of the implants coated with Zirconia and collagen shows numerous bone spicules within an active woven bone, the brown color indicates positive DAB stain (Figure 11).

Six weeks after implantation

A- Uncoated CpTi implants:
Microphotograph view rabbit tibia of uncoated implant site shows blue color of immature bone which indicates negative immunohistochemical stain for OC localization (Figure 12).

B- CpTi implants coated with collagen:
Figure 13, view section of bone of collagen coated implant where the bone is well developed, its blue color indicates negative staining for localization of OC protein.

C- CpTi implants coated with zirconia & collagen (ZEC):
View of negative DAB stain for OC of mature bone, osteocytes are scattered within bone matrix, and formative osteoblast cells are seen at bone surface (Figure 14).
D- Immuno-histochemical examination for growth hormone receptor (GHR) expression of implant in three days, one, two, and six weeks intervals periods:

E- Three days after implantation

F- A- Uncoated CpTi implants:

G- Microphotograph view uncoated implant site after three days of implantation in rabbit tibia shows marrow tissue that stains negatively (Figure 15).

B- CpTi implant coated with collagen:

After three days of implantation primitive osteoid tissue is formed in which numerous number of active progenitor cells are seen, their color indicate positive reaction for GHR localization, few fat cells are shown (Figure 16).

C- CpTi implant coated with zirconia & collagen (ZEC): Primitive bone formation around ZEC coated implant of three days duration of implantation and positive immunohistochemical stain for GHR is seen in progenitor cells and reticular cells, few adipose cells are seen (Figure 17).

One week after implantation

A- Uncoated CpTi implants: View of woven bone formed at uncoated Ti implant site with positive expression of GHR in progenitor cells within the extracellular matrix (Figure 18).

B- CpTi implants coated with collagen: Micrograph view of osteoid tissue formed around Ti implant after one week of implantation, few fibroblast cells and bone formative osteoblast cells are clearly seen (brown color) they show positive DAB stain for GHR, whereas surrounding osteoid tissue stains negatively (Figure 19).

C- CpTi implants coated with zirconia & collagen (ZEC): View of bone section at implant coated with ZEC, in which small areas of primitive bone are stained negatively, surrounded by areas of woven bone tissue with positive DAB stain (Figure 20).
Two weeks after implantation

A- **Uncoated CpTi implants**
Microscopic evaluation of the bone section related to uncoated implant in rabbit tibia after 2 weeks of implantation, show few areas of calcified bone DAB stain (blue color) because of negative stain, this bone is surrounded by positively stained primitive woven bone (Figure 21).

B- **CpTi implants coated with collagen**
View of osteoid tissue shows newly formed bone trabeculae in which osteocytes are embedded, and formative osteoblasts seen rimming bone surface, besides woven bone is formed, all these structures are stained positively except osteoid bone is negatively stained (Figure 22).

C- **CpTi implants coated with zirconia & collagen (ZEC)**
Areas of marrow tissues of different sizes showing positive stain are enclosed by anastomosing trabeculae. Osteoblasts seen at the peripheries and osteocytes are located within bone matrix, both types of cells show positive stain, the trabeculae are stained negatively (Figure 23).

Six weeks after implantation

A- **Uncoated CpTi implants**
Microphotograph view of bone formed around Ti implant (control) at six weeks interval in which bone trabeculae stain negatively for GHR, these trabeculae are enclosing areas of positively stained woven bone (Figure 24).

B- **CpTi implants coated with collagen**
After six weeks of implantation, primitive bone is deposited at the implant site, which shows negative DAB stain for GHR, some osteocytes are seen inside bone matrix, bone forming osteoblasts stain positively (Figure 25).

C- **CpTi implants coated with zirconia & collagen (ZEC)**
Mature lamellated bone is deposited at implant site, it shows negative DAB stain for GHR, some osteocytes are embedded within bone matrix, havercian canals stain positively (Figure 26).
DISCUSSION

Commercially pure (cpTi) titanium is the material most often used in implant manufacturing because of its excellent biocompatibility. Different approaches are being used in an effort to obtain desired outcomes at the bone-implant interface \(^{(12)}\). Many studies have focused on surface characteristics and chemical composition as a way to control bone healing around dental implants \(^{(13)}\). Electrophoretic deposition (EPD) is suitable for the production of thickness-controlled...
laminates with good adherence between the layers (14). A binder is added to increase the adherence and strength of the deposited material and prevent cracking. Polyvinyl butyral(PVB) as a binder, phosphate ester as a dispersant enables formation of stable suspensions and prevents particle agglomeration (15), and ethyl alcohol as a solvent were generally used .The dilution of the sols with ethanol improves their stability reducing the initial values of viscosity and avoiding the agglomeration of particles (16). The radiographic examination in this study, demonstrated a seemingly direct contact between bone and implant, there was no radiolucent zones or any abnormal reaction to the implant. However, the lack of such zones is not evidence for osseointegration, since it is impractical for a clinician to detect changes in the radiographic bone loss at 0.1mm resolution (17). Radiographic examination showed increase in the thickness of cortical bone at experimental implant site indicating increased bone formation and maturation around the coated implants at six weeks duration of implantation. The large increase in torque values between 2 and 6 weeks may be related to the maturation of woven bone to lamellar bone, In rabbits it takes six weeks for the woven bone to be replaced by the lamellar bone with adequate strength for load bearing, a greater removal force is generally interpreted as an increase in the bone healing around the implants and an improved osseointegration (18). Current data suggests a possible role of osteocalcin in body's metabolic regulation and is consider to be preosteoblastic or bone building protein secreated by osteoblast, therefore it is often used as biomarker for bone formation process .The use of zirconia and collagen in coatings of implants showed to act as bone substitutes these substances represent inorganic and major organic components respectively .These two materials enhance mesenchymal stromal cell to differentiate to preosteogenic that secret OC and expressed by osteoblast only it make up of 20% of the non collagenous proteins of the matrix .OC seems to have a role in the early stages of bone formation and some studies (19), suggest that OC is chemotactic for osteoclast and regulate osteoblast activity too.Greater number of positive cells indicate a more rapid tissue reaction on implant surface.Coating titanium surface with collagen enhanced the spreading of cells and speeding cell adhesion length (20). Immunohistochemical findings Osteocalcin (OC):our results shows a greater number of positive cells indicate a more rapid tissue reaction on implant surface. In the present result adipocyte cell type were detected in early period having positive stain with OC while in later period it hardly detected, and it coincides with Song and Tunan, (21) results in transdifferentiation of adipocyte into osteoblast upon exchange of inducing extracellular factors which included using of zirconia and collagen. These materials induce osteogenesis and show a number of lineage in specific program for mesenchymal cell which were in turn upregulate expression of a number of specific gene transcription.

The regulation of hormone and growth factors of different stages of osteoblast differentiation showed differences in expression of osteoblast phenotypic markers by these cells, therefore the results reported positive and negative expression of GHR in the studied periods (3days,1,2, and 6weeks). Uses of different coated of implant (biochemical material) suggest to act as modifying factor for GHR expression that may induce an increase in GHR number expressed on precursor cells and in immature osteoblast while GHR expression decrease in the 6weeks as mature osteoblast present in lamellar bone. Davies, (22) showed that collagen exerts pro-coagulant activity that could also play a significant role, through enhanced platelet activation and concurring release of growth factors; and, as a consequence, in providing the temporary fibrin network matrix required for cell migration to the implant surface.

REFERENCES

Table 1: Two samples statistics for the Removal Torque test in the different studied coating materials after two-six weeks independently

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<th>Comparisons between (2–6) weeks</th>
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<td>Coated with PSZ &amp; Collagen (ZEC)</td>
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