

Does the Coating of Titanium Implants by Hydroxyapatite affect the Elaboration of Free Radicals. An Experimental Study

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Abstract: This study was carried out to throw some light on the mechanisms affecting the reaction of tissue surroundings titanium implants either coated with hydroxyapatite or not, especially the oxidative and nitrooxidative stresses as well as the level of NO in the blood stream. In addition to study the effect of the number of discs on the free radical status in the body and the histological reactions. Forty albino rats were used in this study; they were divided into two groups 20 rats. Group I receiving 50 coated implants with Hydroxyapatite (HA) and was subdivided into G Ia consists of 10 rats receiving one implant and GII b consists of 10 rats receiving four implants. The other group receive 50 uncoated titanium implants; subdivided into G IIa consists of 10 rats receiving one implant and G II b consists of 10 rats receiving four implants. EPR technique using 4-hydroxytempo free radical as an indicator of the balance between all free radical species from one side and NO as well as antioxidants from other side had been used. The results suggest that ROS generated from coated Ti-alloy may be involved in creating appropriate conditions for healing. Moreover; the titanium implants coated with hydroxyapatite leads to the reducing state in the cells faster than that without coating due to inhibition of formation of oxidizing and nitroxidizing species. The histological examination reveals no difference in the capsule thickness of both groups. Conclusions: the coating of titanium implants with hydroxyapatite leads to attaining of reduced state in the cells, which enhance the healing process in comparison with the uncoated implants.

Key words: implants, Hydroxyapatite coated implants, Free radicals, Antioxidants, EPR.

INTRODUCTION

Dental implants have gained steadily increased clinical importance over the last decades, which have led to the rapid development of various different dental implant designs (Rungsiyakull *et al*, 2008). Biomaterial implants are used in a variety of anatomical locations such as dental implants, orthopedic or cardiovascular prosthesis for vessels or heart valves. The importance of the implant surface with respect to tissue reaction has been recognized with regard to maintain a controlled tissue interface (Beumer *et al*, 1994). Commercially pure titanium and titanium alloys including Ti6AL4V that possess high oxidation number and high acidity are widely used in manufacturing of dental and orthopaedic implants. Both mechanical and surface properties of such implants play an important rules in the subsequent biological processes (Rigo *et al*, 2004). The experimental results obtained by numerous investigators (Koklubo *et al*, 2003& Rigoet *al*, 2004), showed that the bioactivity of titanium surfaces is not high enough to induce the direct growth of bone tissue and good bone fixation before several months. So that several efforts were directed to the modification of metal surfaces which are often employed as a mean of controlling tissue – titanium interactions and shortening the time of bone fixation (Koklubo *et al*, 2003). The coating of titanium dental implants with hydroxyapatite (HA) offers the combination between the strength of the metals with the bioactivity of hydroxyapatite ceramic materials (Koklubo *et al*, 2003& Rigoet *al*, 2004). Histological study proved that coated dental implants with hydroxyapatite be highly bioactive with extensive new bone formation and attachment (Lobato *et al*, 2008)

HA-coated implants had the most effect on osteoblastic differentiation, inducing a greater expression of an array of osteogenic markers; the HA coated surface may have a greater ability to enhance osteogenesis (Knabe *et al*, 2004) Furthermore, the HA coating was reported to produce clinical benefits in treatment time reduction and early loading (Iezzi *et al*, 2007). In that respect, several techniques are usually, followed in

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formation of HA coatings, such as sputtering, plasma spray, sol-gel, electrodeposition and biomimetic process (Rigo *et al.*, 2004).

Subcutaneous implantation of different types of implants in rats is frequently accepted method used to evaluate the biocompatibility of biomaterials (Jiang *et al.*, 2002).

Electron Para-magnetic Resonance (EPR) spectroscopy is capable of providing useful physiologic and metabolic (function) information from tissues *in vivo*. Free radicals reactive oxygen species (ROS) are normally present in the body in small numbers. Normal levels of free radical can be beneficial to the body while, excessive free radicals formation causes damage to the cell and tissue. The body naturally protects itself against oxidants by forming antioxidant compounds. Antioxidants play an important role in scavenging oxidants and consequently preventing cell damage (Ho *et al.*, 1970).

It is now established that a balance between bioavailable nitric oxide (NO) concentration and the level of both reactive oxygen and nitroxide species in wounds may be crucial in the repairing process of wounds. The level of NO species was found to be important in such process, since it acts as an antioxidant as well as a scavenger for oxidative radicals such as superoxide. At the same time the high levels of NO strongly affects, positively angiogenesis and endothelial/ skeletal muscle cells, remodeling and proliferation (Dalton *et al.*, 1999, Dissemond *et al.*, 2002 & Sen, 2003).

The damage of the tissue triggers a cascade of repair events which begin with the formation of a fibrin clot. The clot, formed as a result of leakage tissues, serve as a provisional matrix through which cells can move and also as a reservoir for growth factors and cytokines (Clark, 1996). The growth factors initiate the inflammation, epithelialization, wound contraction and angiogenesis process (Jozkiewicz *et al.*, 2004). Platelets derived growth factor (PDGF) and tissue growth factor (TGF) released from platelets act as chemoattractants for neutrophils and monocytes/ macrophages. The major role of neutrophils is to kill the invading microorganisms by their characteristic respiratory burst activity and also to activate keratinocytes and fibroblasts (Sen, 2003). The monocytes in the inflamed tissues can mature into macrophages which are responsible for phagocytosis of dying neutrophils, damaged tissue and microorganisms. Macrophages also play an important role in the long term repair response by releasing a battery of cytokines and growth factors (FGF, PDGF, TGF and TGF β) to amplify the inflammatory response and also to initiate the proliferative phase of wound healing (Clark, 1996). A reepithelialization of wounds is marked by the migration of keratinocytes across the fibrin clot and at the same time of proliferation of keratinocytes at the wound edge. Angiogenesis lead to repuddling of the damaged vessels, restoration of blood flow and restoration of the oxygen supply to the tissue. During the process of angiogenesis, proteases are released from the activated endothelial cells. The proteases are involved in the degradation of endothelial cells and basement membrane. This process allows migration of endothelial cells and their different ion into mature capillary blood vessels (Huk *et al.*, 1997 and Ziche & Morbidelli, 2000).

About 7 days after wounding the clot is completely replaced by fibroblasts that synthesize and remodel a new collagen rich matrix (Sen, 2003). At the same time some proportion of fibroblasts transform into myofibroblasts which resemble smooth muscle and cause wound contraction (Clark, 1996).

The process of implantation of titanium implants leads to creation of a wound at their site of implantation. So that; the objective of this study was to determine and compare the effect of hydroxyapatite coated titanium implants and uncoated titanium implants on the release of ROS in the blood. Furthermore, investigate if the quantity of the inserted implant had any effect or not.

MATERIALS AND METHODS

A total of forty adult albino rats, of average age about 4-6 months and average weight (230-250 gm) were used in this study. All rats were supplied from the same breeder, housed at the same conditions of temperature and humidity in specially designed wire cages in the animal housing of the National Research Centre (NRC).

One hundred pure titanium discs grade 3 constituents (Nitrogen 0.012, carbon 0.032, hydrogen 0.0022, Iron 0.300, oxygen 0.0) substrates (dimensions 1mm thickness and 3mm diameter (MTM-Rifai-Egypt). Half of them were mechanically polished with emery papers to the finest grades, swept with a cotton piece, ultrasonically washed in ethanol and then in distilled water. Some of these substrates were pretreated by 10 M NaOH aqueous solution at 60 °C for 24 h, gently washed with distilled water and left to dry at room temperature. They were washed in deionized water before incubating in simulated body fluid (Rigo *et al.*, 2004)(SBF) to form a coat of calcium phosphate. Samples were immersed into plastic containers with the ratio of sample geometrical surface area to SBF soaking solution. All samples left for 15 days to allow the calcium phosphate formation on the pretreated titanium substrate surface (Mostafa and Attia 2008).

The rats were divided into two groups, each consists of 20 rats. Group I subdivided into: two subgroups. Subgroup Ia 10 rats receiving one implant. Subgroup Ib: 10 rats receiving four implants. Group two consists of 20 rats subdivided into: two subgroups. Subgroup IIa: 10 rats receiving one implant. Subgroup IIb: 10 rats receiving four implants.

Surgical Procedure and Blood Sample Collection:

All animals were anesthetized by intraperitoneal injection of thiopental (30-60 mg kg⁻¹.induction). The procedure was performed under sterile conditions. Base line blood samples were collected from each rat taken from the inner acanthus of the eye in test tube containing 1 ml of 10⁻⁴ molar solution of 4 hydroxy tempo was added to 1 ml of blood samples (anticoagulant EDTA) and the measurement was carried out at room temperature in a silica tube sample for solution. The site which will receive the titanium disc at the back of each rat were shaved and sterilized with betadine then 4 ml incision was made using a sharp scalpel (blade 15). Then a blunt dissection of the skin; inserting the titanium disc subcutaneously and suturing with three 0 black silk sutures were made.

Then blood Samples were taken after 2, 4, 24 and 48 hours postoperatively for each rat by the same researcher.

EPR Measurements:

EPR measurements were measured with Bruker Elex-sys-500 instrument operative at x-band frequency. The process of determination of free radical status in the whole blood of tested rats depends on the reaction of water soluble free radicals, tempol., or hydroxytempo (~ 1 x 10⁻⁴M) with the reducing agents, such as ascorbic acid, nitric oxide (NO). The proposed reaction leads to conversion of tempol free radical to an ESR silent hydroxylamine compound. The area under double integrated signals will be decreased proportionally with increasing the amount of both reducing agents and nitric oxide. On the other hand the increase in the liberated free radicals leads to increase in the areas under peaks in case where their values are higher than the values to be affected by NO and reducing agents.

Histopathological Examination:

Three months later the rats were sacrificed, the implants with the surrounding skin and subcutaneous tissue were retrieved. Then the tissue samples were fixed in 10% buffered neutral formalin.

Histological sections were stained with hematoxylin and eosin; evaluation was carried with respect to cellular response and capsule thickness.

RESULTS AND DISCUSSION

The present study was carried out to evaluate does coating the titanium implants by HA had an effect on the elaboration of ROS and to determine its biocompatibility.

Daily humans use about 250g of oxygen, out of which 2-5% is converted to reactive oxygen species (ROS). The last term means that all oxygen associated species that have higher oxidative potential (i.e. higher activity) than molecular oxygen: singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical (Dissemond *et al*, 2002). In the ground state, molecular oxygen is being found in a relatively inert triplet state ³O₂. The initial event that activates oxygen in biological systems is a change of electron spin pairing. This change results from one of at least three different chemical mechanisms:

The first mechanism involves an elevation of one electron to a higher energy level and production of unpaired electrons with antiparallel spins. By this mechanism organic endoperoxides and UV or near UV radiation in combination with the photosensitizing chemicals produce singlet oxygen (¹O₂). The second mechanism involves reduction of one molecule of oxygen by one electron, where transition metals and organic electron donors reduce triplet oxygen (³O₂) by this mechanism and produce superoxide (O₂⁻) and metal-oxygen complexes such as perferryl and related species. The third mechanism involves abstraction of one electron (or hydrogen) from an organic compound. In this manner the carbon radicals (>C<) resulting from hydrogen abstraction by hydroxyl radical (HO[•]) react with ³O₂ and produce peroxy radicals (OOH[•]).

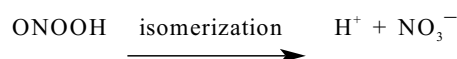
The superoxide can rapidly dismutate to ³O₂ and H₂O₂ by means of superoxide dismutase enzyme (SOD). H₂O₂ can also be detoxified by catalase enzyme to H₂O and ³O₂. The toxicity of superoxide expressed itself in its capability for formation of toxic hydroxyl radical as (2OH[•]) as a result of interaction with transition metals especially ferrous ion (Huk *et al*, 1997). The last interaction can be expressed according to the following equation,



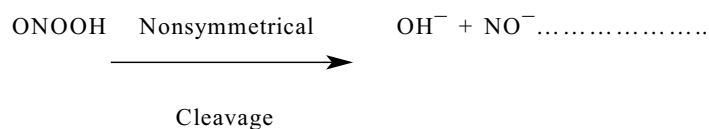
The hydroxyl free radicals are very reactive, where it can react with another chemical species within five molecular diameters from its point of origin ⁽¹⁵⁾. The previous oxidizing species are considered as strong oxidants and can contribute to oxidative damage in cells (Dalton *et al*, 1999) . Due to such effect a delicate balance is found in biological systems between an amount of oxidants and antioxidants for prevention of oxidative damage to cells. The oxidants have the favour that they play an important role in wound healing (Ziche& Morbidelli, 2000) providing signaling and defense against microorganisms. However; the oxidants have to be detoxified in order to prevent damage of host cells. The antioxidant defense system involves reduction (scavenging) and / or dismutation of O₂⁻ and their protonated forms. When the antioxidant defense system fails to eliminate the oxidants; the alteration in homeostasis leads to oxidative stresses which favor the damage of the cell.

The liberation of reactive oxygen species during different stages of wound repair, leads also, to the formation of several nitrogen compounds which are known as nitroxidative stress (Jozkowicz *et al*, 2004). These compounds namely are peroxyxynitrite anion (ONOO⁻), peroxyxynitrous acid (ONOOH), nitronium ion NO₂⁺, nitrogen dioxide radical (NO₂[·]), nitryl chloride (NO₂CL) and nitrite anion (NO₂⁻). The previous nitroxidative species can be formed as a result of rapid oxidation of NO, i.e. it reacts with O₂⁻ and O₂ leading to formation of NO₂⁻, ONOO⁻ and NO₂. The last three ones can be transformed to NO₂CL; CO₃⁻, NO₂, N₂O₃ via reaction with HOCl, CO₂ and NO respectively (Brovkovich *et al*,1999, Huk *et al.*, 2000, Kalinowski 2000 and Klatt & Lamas 2000).

At low concentrations ONOO⁻ can isomerize to the harmless nitrate ion according to the following equation.,



However when nitrogen compound generated at high concentration (>30-50 mmol/L) peroxyxynitrite can diffuse and during the diffusion process, where it can undergo symmetrical or non – symmetrical cleavage to form NO₂⁻ and OH[·] or NO₂⁺ and OH⁻ respectively,



Three of the resulted species are powerful oxidants and contribute to increase in oxiatative (OH[·]) and nitroxidative (OH[·] + NO₂⁺) stresses ⁽¹⁹⁾. In addition, peroxyxynitrite can also react with carbonate ion to form nitroso-peroxocarbonates. The putative intermediate, 1- carboxylato-2- nitrosodioxidane, fragments into the free radicals trioxocarbonate (CO₃⁻) and nitrogen dioxide (NO₂[·]). The last two radicals can, also, damage different biomolecules via processes of oxygenation, nitrosation and nitration of them (Kirsch & De Grost, 2001).

Antioxidants are important to maintain low levels of free radicals and reactive nonradical species derived from radicals. These includes enzymes SOD, glutathione peroxidase , and catalase and also nonenzymatic compounds such as vitamin E, b- carotine , ascorbate and glutathione, zinc and selenium. These antioxidants may have a role in quenching the oxidants as there is high level of protease activity in early phases of wound healing. The most effective antioxidant is NO, which is a scavenger of superoxide. However, the process of scavenging superoxide by NO decreases the oxidative stress while increasing the nitroxidative stress (Pryor *et al*, 1995, Barrick *et al*, 1999 and Droge, 2002).

The reactive oxygen species preoxyxynitrite (OONO⁻) has been known to play a role in inflammation and considered as a potent oxidant capable of a wide range of reactions (Pryor & Squadrito, 1995). Clinical studies also provide evidence that peroxtnitrite is produced during inflammation. The blood serum and synovial fluid from patients with the inflammatory joint disease were found to contain 3-nitrotyrosine markers, indicating

peroxynitrite formation while body fluids from normal patients contained no detectable 3-nitrotyrosine. Similarly, no 3-nitrotyrosine markers were detected in body fluids from patients with osteoarthritis, a largely non-inflammatory joint disease (- Kaur& Halliwell, 1994).

In that respect, it has been reported that 3-nitrotyrosine markers for peroxynitrite also have been observed at the interface membrane for hip implants suffering from aseptic loosening, which is characterized by local inflammation (Hukkanen *et al*, 1998).

The results presented in Table (1) showed that the EPR signal for all free radical was found to be increased by increasing the time during the early stages of implantation to 24h.

Table 1: Showed the mean and std deviation of the level of NO in both groups at different time intervals

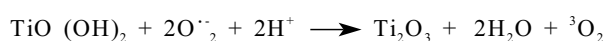
Time intervals	Group I Coated implants	Group II Uncoated implants
Control	26.40±5	19.86±4
0 h	14.55±2.3	20.34±11.5
2 h	6.88±4.2	19.31±14.5
4 h	13.50±7.8	3278±10.5
24 h	11.86±6.3	22.06±5.2
48 h	4.82±2	9.33±3.7
P value	0.001	
Significant	Sig	

(0) h increase in the free radicals others than NO species (Increase in ·OH, ·O₂⁻ radical is higher than liberation of NO species.
 24 h increase in ·OH, ·O₂⁻ radicals is higher than liberation of NO species., 48 h increase in ·OH and ·O₂⁻ radicals .
 Increase in NO starting from (0) h with respect to other free radicals

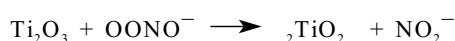
This finding may be as a result of the increase of vital processes which lead to increase in the formation of free radicals, e.g. singlet oxygen, superoxide anion, carbon, hydroxyl and NO·₂ radicals.

By increasing the time the species of nitrooxidative stress seems to be increased in the blood stream as a result of oxidation of nitric oxide species to peroxynitrous acid together with NO₂⁺, NO·₂, NO₂Cl and NO·₂ according to the manner described before.

The manner by which titanium reduces, to a great extent, the harmful oxides is rather complex. In that respect one can consider that the formed tetravalent, Ti⁴⁺, on the surface of implant is considered as a hard acid and has higher affinity towards hard bases such as anions of F⁻, OH⁻ and Cl⁻. S⁺⁶ that the outer surface suffers from stability and react with hydroxyl ion leading to formation of TiO (OH)₂ compound. The last compound reduces the level of superoxide via transformation of the last species to ³O₂ (triplet oxygen) according to the following eqn.,



The resulted trivalent titanium ion is considered as a less harder acid than tetravalent one so that it is assumed that it can react with peroxynitrite anion leading to reproduction of tetravalent titanium ion and reducing nitrite one according to the following eqn.,



The last two reactions; leads to reduction of both oxidative and nitrooxidative stress.

The last suggestion reflects itself in the pronounced decrease in the amount of free radicals and increase in NO and antioxidants in the blood, stream after 48h. Moreover, the results clarified that the implants coated with a thin layer of hydroxyapatite showed a pronounced depression in the content of free radicals and a pronounced increase in the liberation of NO species (Table 1).

It seems that hydroxyapatite inhibit the formation of oxidative and nitrooxidative species according to the following suggestions,

- a- It seems that hydroxyapatite favors the isomerization of peroxynitrous acid (ONOOH) to nitric acid.
- b- It favors the nonsymmetrical cleavage of ONOOH yielding OH⁻ and NO₂⁻ , radicals, and inhibit the symmetrical cleavage which leads to formation of OH· and NO·₂ radicals.
- c- It inhibits the transformation of ONOOH to CO₃⁻ and NO₂ via reaction with CO₂.

As seen in Table (2) there was a significant differences in the antioxidant status between the coated and uncoated implants groups. However; as regard the number of the implants inserted there was no significant difference between one implant and four implants in both groups.

Table 2: Showed the level of NO in the blood of the two groups

No of implant	Group I coated implant	Group II uncoated implant	P value	Significant
One implant	13.87 ± 8.1	23.10± 12.3	0.041	Sig
Four implants	12.43 ± 9.5	18.13 ± 8.9	0.160	N S

From the abovementioned results it's obviously safe to insert more than one implant at the same visit which wouldn't lead to cause any increase in the level of ROS especially in coated implants which exhibits a positive level of antioxidants more than the uncoated group.

A balance between available nitric oxide concentration from one side and the level of both oxidative and nitrooxidative stresses in wound healing may be crucial in wound repair .The highly beneficial effect of bioavailable, no is attributed to the scavenging of superoxide, which is the main component of oxidative stress. On the other side the high level of NO can influence positively angiogenesis and endothelial skeletal muscle cell remodeling and proliferation.

None of the tissue specimens showed any evidence of immune or inflammatory responses to the installed implants whether coated or uncoated. All implants were encapsulated by fibrous connective tissue consisting of fibroblasts, few inflammatory cells and collagen fibers with no difference in the capsule thickness around coated and uncoated implants (Fig 1,2)

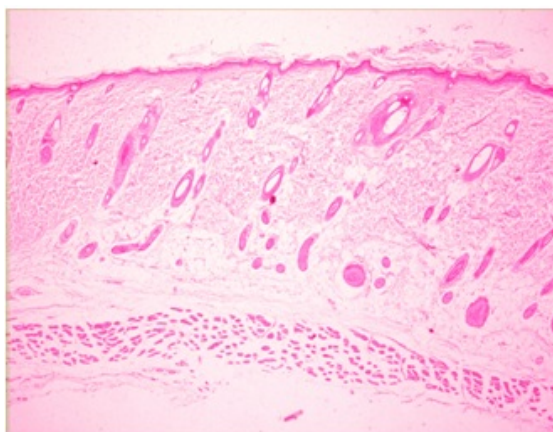


Fig. 1: Photomicrograph showing the different layers that appear as normal (H&Ex100)

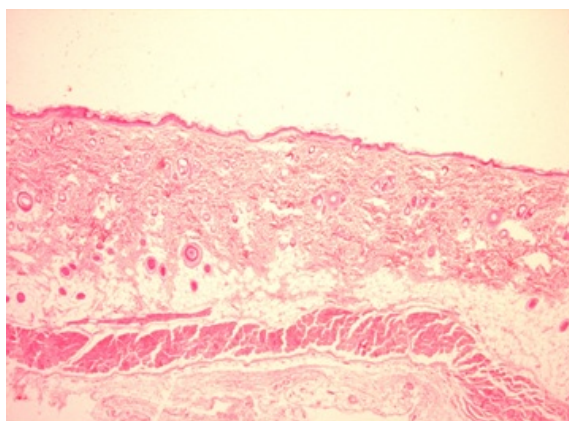


Fig. 2: Photomicrograph showing normal epidermis and dermis with collection of fibrous tissue under the dermis(H&Ex100)

Conclusion:

None of the rats showed any evidence of immune or inflammation response towards titanium alloy whether coated with hydroxyapatite or not.

The technique followed in the present investigation can be used in case of tracing of wound healing since it shows the status of free radicals as well as NO species in blood stream.

The results showed that the coating of titanium implants with hydroxyapatite leads to attaining of reduced state in the cells, which enhance the healing process in comparison with the uncoated implants.

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