Decision Letter (XRS-13-0070.R1)

From: rene.vangrieken@uantwerpen.be

To: jabraham.famaf@gmail.com, jsan@famaf.unc.edu.ar, mirigren@odo.unc.edu.ar, perez@lnls.br

CC:

Subject: X-Ray Spectrometry - Decision on Manuscript ID XRS-13-0070.R1

Body: 17-Feb-2014

Dear Prof. Sánchez,

It is a pleasure to accept your manuscript entitled "TXRF Analysis of Metals in Oral Fluids of Patients with Dental Implants" in its current form for publication in X-Ray Spectrometry.

Your article cannot be published until the publisher has received the appropriate signed license agreement. Within the next few days the corresponding author will receive an email from Wiley's Author Services system which will ask them to log in and will present them with the appropriate license for completion.

Thank you for your fine contribution.

Sincerely,

Dr. Rene Van Grieken
Editor-in-Chief, X-Ray Spectrometry
rene.vangrieken@uantwerpen.be

Date Sent: 17-Feb-2014

Close Window

X-Ray Spectrometry

TXRF Analysis of Metals in Oral Fluids of Patients with Dental Implants

Journal:	*X-Ray Spectrometry*
Manuscript ID:	XRS-13-0070.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	17-Feb-2014
Complete List of Authors:	Abraham, José; Universidad Nacional de Córdoba, Facultad de Matemática Astronomía y Física Sánchez, Héctor; Universidad Nacional de Córdoba, Facultad de Matemática Astronomía y Fisica Grenón, Miriam; Universidad Nacional de Córdoba, Facultad de Odontología Pérez, Carlos; Brazilian Synchrotron Light Source, Fluorescence and Absorption group
Keywords:	TXRF, Dental implants, Metal traces, Synchrotron Radiation

TXRF Analysis of Metals in Oral Fluids of Patients with Dental Implants

José A. Abraham[1], Héctor J. Sánchez[1], Miriam S. Grenón[2], and Carlos A. Pérez[3]

[1] Facultad de Matemática Astronomía y Física, Universidad Nacional de Córdoba, Argentina

[2] Facultad de Odontología, Universidad Nacional de Córdoba, Argentina

[3] Laboratorio Nacional de Luz Sincrotron, Campinas, Brazil

Abstract

Corrosion of metals in implanted biomaterials lifetime is expected to occur. Nowadays medical implants have good biocompatibility, present proper mechanical properties, and promote tissue regeneration; nevertheless, corrosion will eventually happen. Biological fluids are rich in chemically active ions, hence, electrochemical processes appear on the surface of the metal immediately after implantation. In order to evaluate corrosion resistance of metal implants, several studies have been carried out in artificial environments but their results have not been always directly correlated to living systems.

This work presents an indirect study of corrosion of dental implants by analyzing changes of elemental concentration of metals in oral fluids. It will also contribute to the knowledge of implant corrosion in relation to its biological environment. Degradation of the implant surface releases material to the medium, which depending on the concentrations can represent toxic risk, organic malfunction, pain, rejection, etc. In order to evaluate this process, the concentrations of representative metals such as Ti, Al, and V in saliva and gingival fluids were analyzed by means of total reflection of x-rays fluorescence analysis using synchrotron radiation.

The results obtained here show that Ti-ions present a different behavior in the oral fluids, revealing higher concentrations in gingival crevice fluid than in saliva. On the other hand, V and Al have not shown significant differences from normal levels in the oral fluids. Metal release is discussed under mechanical and chemical considerations, taking into account the oral environment of the implant.

Introduction

Materials used in osseous implants have a significant relevance. The implant material must be inert in the living medium and must not suffer any kind of deterioration by chemical or biological agents. There are several materials that fulfill these requirements; gold and silver are good examples of that, but they are too expensive. Titanium (and/or Ti-alloys) represents a proper alternative. Nowadays the use of this metal as dental implant material is a common solution for dental restitution. Titanium has good mechanical properties and low density; it is also abundant and not expensive. Titanium implants accomplish the

requirements of biocompatibility and present good conditions to promote the growing of new tissue on its surface.

Some characteristics of the implant surface can improve the adaptation to the bone. For instance, the formation of titanium dioxide on the surface of a dental implant favors hard tissue adhesion to the implant [1] and prevents chemical attack by external reagents. This TiO_2 layer is called a "passive layer". Surface morphology is also important to improve the adsorption of plasma proteins; for instance, albumin and IgB develop preferably on rough surfaces of titanium [2]. In addition, free ions in body fluids are involved in electrochemical processes producing corrosion, and gradual degradation of metallic constituents.

Body fluids (especially the oral ones) are sensitive to hormonal and inorganic alterations [3], making this kind of sample very suitable for systemic diagnosis. From the medical point of view, saliva and gingival fluids have advantageous characteristics: the extraction methodology is neither invasive nor painful, the procedure is not expensive, and it presents a low probability of infection. Because of these properties, it is possible to implement analytical tests on medical laboratories for evaluating metal contamination [4,5] or corrosion of implanted material.

After implantation, titanium implants are exposed to the periodontal physiology allowing new bone formation. Immediately after implantation, electrochemical activities of free ions and bacteria bioactivity on the interface metal-fluid begin. Nevertheless corrosion takes place after a long period of time, maybe years, because of the titanium passive barrier on the surface. After this latency period, and under certain chemical (pH, electrolyte concentrations, etc.) and mechanical conditions (wear, stress, etc), the passive layer deteriorates and corrosion begins, varying the normal concentrations of this metal in different organs and fluids. The influence and extent of this contamination is not well-know yet.

The variations of metallic-ion levels can be studied in the mineral and the organic components. The release of metals due to corrosion has a direct impact on the mineral contents of systemic fluids i.e. saliva, gingival crevice fluid and blood. Systemic changes can be studied through these changes [6]. Abnormal metal concentrations can also be perceived by organic changes at levels of proteins and enzymes. For example, hexavalent chromium ions are released from implant materials causing lipid peroxidation [7]. Ni and Cr [8] induce Type-IV hypersensitivity reactions in the body and act as haptens, mutagens, and carcinogens. They produce cytotoxic responses including a decrease of some enzyme activities, interference with biochemical pathways, mutagenicity, and carcinogenicity. Manganese contained in alloys can also be detected in saliva. Manganese toxicity produces skeletal and nervous system disorders. Titanium ions have not shown evidence of causing toxicity in tissue, but they can initiate other unwanted processes related to loss bone and osteolysis. Osseointegration is especially affected by corrosion and its waste products such as Al and V. According to Roynesdal, et al. [9], marginal-bone loss around implants produces osseointagration failure with titanium-sprayed implants. Olmedo, et al. [10] reported that the presence of macrophages in peri-implant soft tissue induced by a corrosion process plays a central role in implant failure. Free titanium ions inhibit growth of hydroxyapatite crystals on

surface implants. These processes end up in local osteolysis and loss of clinical stability of the implant.

To summarize, metal ion release from implants is known to occur, but most data reported in the literature are mainly related to *in vitro* studies, which have not always a direct implication on living systems. Measurements in real systems are scarcely reported despite of toxic risk of some metals in body fluids at abnormal levels. Metal degradation in oral fluids is expected because saliva is a chemically aggressive environment. In general, the study of corrosion in living system will provide useful information for physician to make a suitable choice, and for manufacturers to improve their products.

The aim of this work is to determine the Ti, V and Al concentration in saliva and gingival crevice fluid of patients with dental implants by total-reflection x-ray fluorescence (TXRF) analysis using synchrotron radiation. The measurements were carried out for pure titanium and for a Ti-Al6-V4 alloy. These results will contribute to evaluate the procedure as a means of determining corrosion in progress, and to assess if the level of these metals in fluids are high enough to consider risk of toxicity. To our better knowledge, these results have not been reported before.

Experimental

The samples were extracted from 49 patients who required attention at the dental care center and agreed to participate in the investigation. They were divided in two groups, 23 subjects with dental implants (the experimental group) and 26 subjects without dental implants (the control group). In both cases, the subjects were selected according to the following criteria:

Inclusion criteria

- Adults of both sexes.
- Two or more functional dental areas.
- Have not used toothpaste for oral hygiene since two days before sampling.
- Dental implant two years old or more.
- Underwent periodontal treatment within last year.

Exclusion criteria

- Systematic pathologies.
- Receiving regular medication or treatment.
- Smokers.
- Have extra-oral metallic implant in the body.

Samples of saliva were taken from the mouth floor and gingival fluid samples were extracted from the gingival area of lower incisors; they were collected by using calibrated microcapillaries. These calibrated microcapillaries are commercially available, they are 5 cm

long, the internal diameter is 10 microns and the external diameter is 500 microns. The amount of liquid collected is obtained by measuring the length of the liquid inside the capillary. Using micropippettes, the samples were deposited on flat-polished acrylic supports, and allowed to dry in air; the sample volume ranged between 1 µl and 5 µl. An internal standard was used for quantification, which consisted of a solution of 998 ppm of gallium diluted in 3 ml of tri-distilled water, 1 µl droplet was spilled on the reflectors.

Usually, the internal standard is mixed with the sample before it is dropped on the reflector. In this case the amount of collected samples is so small that mixing is not possible. An observation under the microscope shows a good homogenization. Even so, the size of the irradiation beam was adjusted in order to cover the whole area of the sample on the reflector. Regarding the internal standard (gallium), it is a typical element added for TXRF quantification because it does not interfere with other elements of usual interest and cover a wide range of elements to be quantified.

Commercially, several materials are used for medical implants. They are based on steels, platinum or titanium alloys. Titanium based alloys are widely used in dental implants. Patients participating in this work had a dental implant based on a Ti–Al6–V4 alloy, which is a widely-used commercial alloy composed of titanium, 6% of aluminum, and 4% of vanadium. All the implantations dated more than two years old and were implanted at the same dental office following the same procedure.

The measurements were carried out in the TXRF station of the D09B beamline at the LNLS (Campinas, Brazil) [11]. The measurements were performed using synchrotron white beam in order to irradiate the element of interest (Ti) and the standard (Ga) efficiently, but also other elements of interest such as Ca, V, Cr, Mn, Al, S, P. The detection system consisted of a Si(Li) solid-state detector with a Be window of 25 µm located at 5 mm from the sample holder with an energy resolution of 148 eV at 5.9 keV, the data acquisition system consisted of a fast amplifier and a multichannel analyzer with an ADC of 25 µsec of conversion time. In order to keep the detection limits under reasonable values (less than 10 %), a collimator of 1 mm in diameter was located in front of the detector window. The experiments were carried out in air atmosphere and the measuring time was of 300 s (live time) for each sample. A scheme of the experimental setup is shown in Figure 1.

Experimental evaluations of the minimum detection levels for theses samples in theses experimental conditions indicate that MDL are 0.5 µg/ml for Al, 0.1 µg/ml for Ti, and 0.04 µg/ml for V,

Data Analysis and Results

Figure 2a-b show typical spectra of gingival crevice fluid and saliva, indicating the peaks of the elements of interest i.e., Ti, V and Al. The measured spectra were analyzed with the AXIL software package [12] taking into account escape peaks, sum peaks and representing the background by a linear function. The reduced-χ^2 of the fittings was 1.2±0.2

and the statistical errors of the calculated intensities were less than 2 % for the strongest lines. Concentrations were estimated by using sensitivities applying the standard procedure [13].

Titanium concentrations in each sample were determined with an error lesser than 7% calculated by propagation. Average values of titanium concentrations for saliva and gingival crevice fluid are shown in Figure 3 and the concentrations for all the elements are compiled in Table I. Uncertainties represent the statistical deviation of the average values. These values show statistically significant differences, $P<0.05$, in titanium concentrations for gingival crevice fluid in the experimental group (22±7) μg/ml with respect to the control group (1.3±0.4) μg/ml. On the other hand, there were not statistical differences of titanium concentration levels in saliva when both groups were compared. The obtained Ti contents were (2.5±0.5) μg/ml and (2.8±0.5) μg/ml for control and experimental group respectively, which were not statistically different at $P>0.05$. All the statistical calculations were carried out according to the one-way ANOVA analysis [14]. According to this analysis, the significant difference between the mean elemental concentrations of titanium in gingival fluid for the experimental group vs. the control group is due to the presence of the implant.

In addition, the contents of vanadium and aluminum in saliva and gingival fluid were analyzed in order to verify if these elements were also released from the implant alloy. In order to study aluminum levels, smoker patients were rejected because the concentration level for this metal is high in saliva as it was recently reported by Kim et al.[15]. The Al concentrations measured in saliva were (6±5) μg/ml and (4±3) μg/ml for control and experimental group respectively. In gingival fluid samples the Al concentrations measured were (5±4) μg/ml and (3±2) μg/ml for control and experimental group respectively. Concentration levels of vanadium in saliva were (0.5±0.4) μg/ml and (0.07±0.06) μg/ml for the control and the experimental group respectively. In gingival fluid samples the results were (0.1±0.1) μg/ml and (0.08±0.07) μg/ml for the control and the experimental group respectively. For all the pairs of measurements, the mean values were not significantly different, with $P>0.05$.

Discussion

Studying implants corrosion is important in order to understand the chemical and physical interaction between implants and the biological surroundings as well as the necessary conditions for this to happen. Experiments in simulated environment with artificial body fluids are not always applicable to real cases. The clue is to analyze biological samples which have been in contact with substances in corrosion process.

The mechanism of corrosion in biological surroundings can be initiated or propitiated by several reasons, which leads to pitting, crevice, and fretting corrosion, among others processes. Depending on the implantation position, one of these mechanisms will take place

preferentially; for example, fretting corrosion is a problem in hip, knee and shoulders prostheses because mobile parts are in constant friction due to the articulation.

Dental implants allow investigating the release of metals in vivo by taking samples directly from mouth. No conclusive results about concentrations of released metal have been obtained in serum [15], blood [16] and urine [17], possibly due to the different mechanisms of absorbing and metabolizing metals like Ti, Co, V etc. Taking saliva or gingival fluid samples represent an alternative way to quantify metal release since these metals are not involved in the bioorganic processes of the mouth.

Ti-based alloys have good anti-corrosive properties in several environments due to their velocity to form passive layers. Nevertheless, in some conditions they have shown crevice corrosion. For example, cemented (Ti–Al6–V4)-hip implants present corrosion caused by the hydrolysis of dissolved metal ions in the crevice region between the metal and bone [15]. The results presented in this work indicate that a similar effect is taking place in dental implants. Titanium concentrations in gingival fluids of patients with implants have shown very high values (22±7) µg/ml with respect to control subjects (1.3±0.4) µg/ml. The increased concentrations of Ti come, undoubtedly, from the implants because there is no other source of Ti-ions, according the exclusion criteria mentioned previously. On the other hand, no statistically significant differences were observed for the concentration of Ti in saliva between the experimental group (2.8±0.5 µg/ml) and the control group (2.5±0.5 µg/ml). This fact indicates that the ions released by the implants are incorporated to the organism via the gingival crevice fluid and metabolized in the organs not reaching the salivary glands.

Several sources of corrosion have been suggested, all of them lead to dissolution of the tiny surface layer of TiO_2. In the oral environment, saliva with native salts operates as weak electrolyte; its action on the TiO_2 layer is enhanced by ions concentrations, pH, buffering capacity and surface tension [16]. Dental implants are included in this scenario because gingival crevice fluids fill the interstitial space between implant and bone. These fluids contain F^- and Cl^- ions, radicals and related acids, which eventually may reach critical values to start corrosion [17].

Aluminum and vanadium have no major relevance because they are minor components in implants. In addition, some authors have reported that the release rate of V and Al are one order of magnitude lower than Ti release rate [18]. Hence, it can be assumed that the released amount of these metals makes no substantial contribution to the normal levels. The results obtained in this work for V and Al in the implanted group are in agreement with this assumption, since they are not statistically different from normal values. Nevertheless statistical variations are observed due to dietary sources (i.e., vegetables have large concentrations of V [19]) and environmental sources such water and air quality. It should be noted that typical V and Al concentrations in oral fluids are very low. Such detections limits can only be reached with TXRF using synchrotron radiation (like in this work), inductively coupled plasma (ICP) [20] (100 ng/L of Al in saliva), and electrothermal atomic absorption spectrometry technique (ETAAS) [21] (1ng/L of V in saliva).

Conclusions

There are many studies about Ti corrosion *in-vitro* or in artificial environments, nevertheless very few results were accomplished in real environments. This work presents new and interesting results obtained directly from the biological samples in real environment.

These results indicate that, even when dental implants are made of a Ti alloy or commercially pure titanium of grade 2, the material suffers some kind of corrosion or degradation, delivering Ti-ions to the sulcus. This is concluded by observing that Ti content in saliva remained close to normal values (1.3±0.5) μg/ml while Ti concentrations in gingival fluids increased up to (22±7) μg/ml, showing a very contrasting feature.

This work shows that synchrotron radiation induced TXRF can be employed to analyze saliva and gingival fluids. TXRF with synchrotron radiation proved to be a precise and reliable method of analysis of this kind of samples, with high sensitivity and low detection limits. This technique can contribute greatly to identify or to predict several oral or systemic severe illnesses. Moreover, standard TXRF is also adequate because the detected levels of Ti can also be reached with conventional x-ray sources.

Acknowledgement

This work was partially supported by the LNLS, Campinas-Brazil.

References

1 A. Schroeder, E. Van der Zypen, H. Stich, and F. Sutter, "The reactions of bone, connective tissue, and epithelium to endosteal implants with titanium-sprayed surfaces", *J. Maxilofacial Surg.* **9**:15–25, (1981).

2 M.N. Sela, L. Badihi, G. Rosen, D. Steinberg, D. Kohavi , "Adsorption of human plasma proteins to modified titanium surfaces", *Clin. Oral Impl. Res.* **18**:630–8, (2007).

3 S. Chiappin, G. Antonelli, R. Gatti and E. F. De Palo, "Saliva specimen: A new laboratory tool for diagnostic and basic investigation", *Clin. Chim. Acta* **383**:30–40, (2007).

4 G.R. Costa de Almeida, C.U. de Freitas, F. Barbosa Jr., J.E. Tanus-Santos, R.F. Gerlac, "Lead in saliva from lead-exposed and unexposed children", *Sci Total Environ.* **407**(5):1547–50, (2009).

5 M.C. Talio, M.O. Luconi, A.N. Masi and L.P. Fernández, "Cadmium monitoring in saliva and urine as indicator of smoking addiction", *Sci. Total Environ*. **408**(16):3125–32, (2010).

6 F. Gil, A. F. Hernández, C. Márquez, P. Femia, P. Olmedo, O.López-Guarnido, A. Pla, "Biomonitorization of cadmium, chromium, manganese, nickel and lead in whole blood, urine, axillary hair and saliva in an occupationally exposed population", *Sci. Total Environ.* **409**(6):1172–80 (2011).

7 M. Sugiyama, "Role of cellular antioxidants in metal-induced damage", Cell Biol. Toxicology **10**:1–22 (1994).

8 M. Cortada, L. Giner, S. Costa, F.J. Gil, D. Rodriguez, J.A. Planell. "Galvanic Corrosion behaviour of titanium implants coupled to dental alloys". *J Mater Sci: Mater. Med.* **11**:287–93, (2000).

9 A.K. Røynesdal, E. Ambjørnsen, H.R. Haanaes. "A Comparision of 3 different Endogenous nonsubmerged implants in endentoulous mandible: A clinical report". *Int. J. Oral Maxillofac. Implants*. **14**:543–8 (1999).

10 D. Olmedo, M.M. Fernadez, M.B. Guglidmotti, R.L. Cabrini. "Macrophages related to dental implant failure". *Implant Dent*. **12**:75–80 (2003).

11 C.A. Pérez, M. Radtke,H.J. Sánchez,H.C.N. Tolentino, R.T. Neuenshwander, W. Barg, M. Rubio, M.I.S. Bueno, I.M. Raimundo, J.J.R. Rohwedder, "Synchrotron radiation X-ray fluorescence at the LNLS: beamline instrumentation and experiments", *X-ray Spectrom.* **28**:320–6, (1999).

12 P. Van Espen, H. Nullens and F. Adams, "A computer analysis of X-ray fluorescence spectra", *Nuc. Inst. & Meth*. **142**:243–250, (1977).

13 R. van Grieken, A. Markowicz (Eds.), Handbook of X-Ray Spectrometry: Methods and Techniques, Marcel Dekker, New York, 1993.

14 J.H. McDonald, Handbook of Biological Statistics. pp 119, Sparky House Publishing, Baltimore, Maryland, (2008).

15 Y-J Kim, Y-K Kim and H-S Kho, "Effects of smoking on trace metal levels in saliva", *Oral Diseases* **16**(8):823–830 (2010).

16 Jacobs JJ, Gilbert JL, Urbani RM. "Corrosion of metal orthopedic implants", *J. Bone Joint Surg. Am.*; **80**:268–82, (1988).

17 N. Schiff, B. Grosgogeata, M. Lissaca and F. Dalardb, "Influence of fluoride content and pH on the corrosion resistance of titanium and its alloys", *Biomaterials*; **23**(9): 1995–2002, (2002).

18 Y. Okazakia, E. Gotoh, "Comparison of metal release from various metallic biomaterials in vitro", *Biomaterials*; **26**:11–21, (2005).

19 S. Al-Omari "Determination of essential and toxic elements in ten herbal medicines using energy-dispersive XRF analysis", *X-Ray Spectrom.*; **40**:31–36, (2011)

20 Pacheco P., Gil R., Smichowski P., Polla G., Martinez L., "Trace aluminium determination in biological samples after microwave digestion followed by solid phase extraction with L-methionine on controlled pore glass", *Microchemical Journal* **89**:1–6, (2008).

21 Berton P., Martinis E.M., Wuilloud R.G., "Development of an on-line temperature-assisted ionic liquid dispersive microextraction system for sensitive determination of

vanadium in environmental and biological samples", Journal of Hazardous Materials **176**:721–728, (2010).

Figure Captions

Table I. Average titanium, vanadium and aluminum concentrations (in μg/ml) for saliva and gingival crevice fluid of the group of patients with dental implants (23) and the control group (26).

Figure 1. A schematic view of the TXRF setup mounted in the D09B beamline of the LNLS.

Figure 2a. Typical x-ray fluorescence spectra of saliva from control and implanted groups. If not specified, all the indicated analytical lines correspond to the Kα ones.

Figure 2b. Typical x-ray fluorescence spectra of gingival fluid from control and implanted groups. Note the different intensity (more than one order of magnitude) of the Ti Kα lines.

Figure 3. Bar graph of average titanium concentrations for saliva and gingival crevice fluid of the group of patients with dental implants and the control group.

Table I

Sample Type	Saliva			Gingival Crevice Fluid		
Elements	**Ti**	**V**	**Al**	**Ti**	**V**	**Al**
No-Implant	2.5 ± 0.5	0.5 ± 0.4	6 ± 5	1.3 ± 0.4	0.1 ± 0.1	5 ± 4
Implant	2.8 ± 0.4	0.07±0.06	4 ± 3	22 ± 7	0.08±0.07	3 ± 2

Average titanium, vanadium and aluminum concentrations (in µg/ml) for saliva and gingival crevice fluid of the group of patients with dental implants (23) and the control group (26).

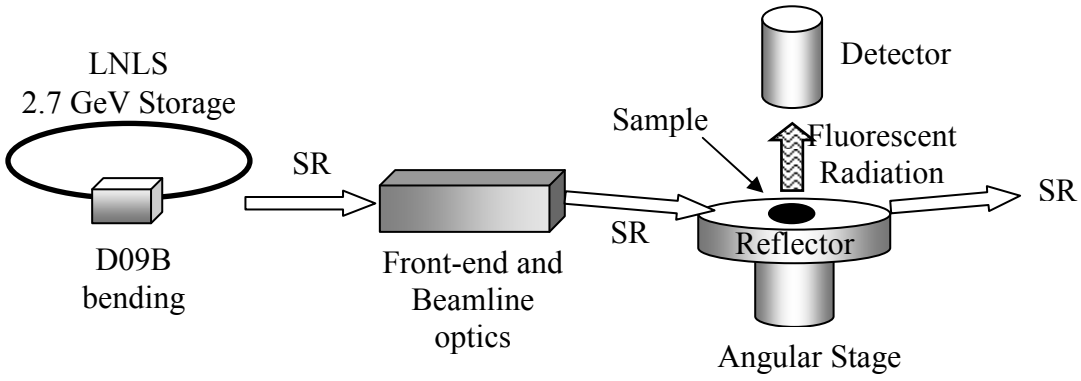

Figure 1. A schematic view of the TXRF setup mounted in the D09B beamline of the LNLS.

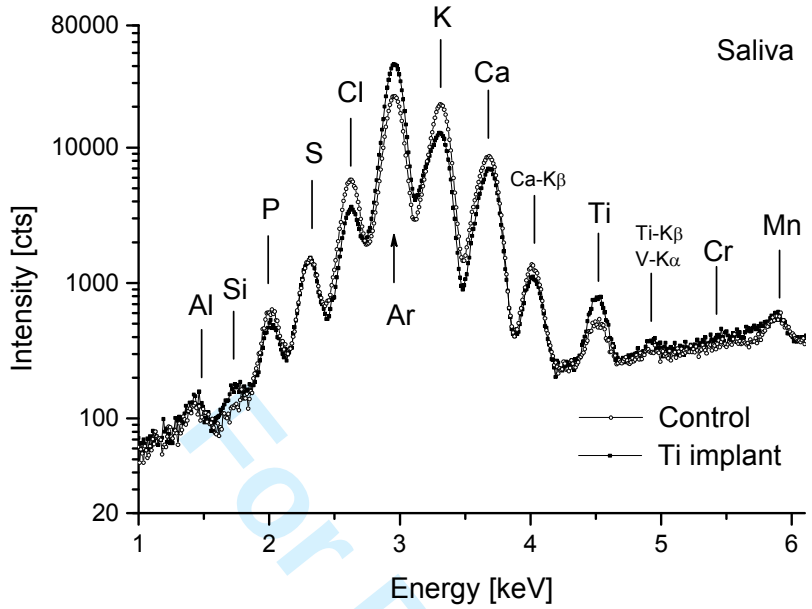

Figure 2a. Typical x-ray fluorescence spectra of saliva from control and implanted groups. If not specified, all the indicated analytical lines correspond to the Kα ones.

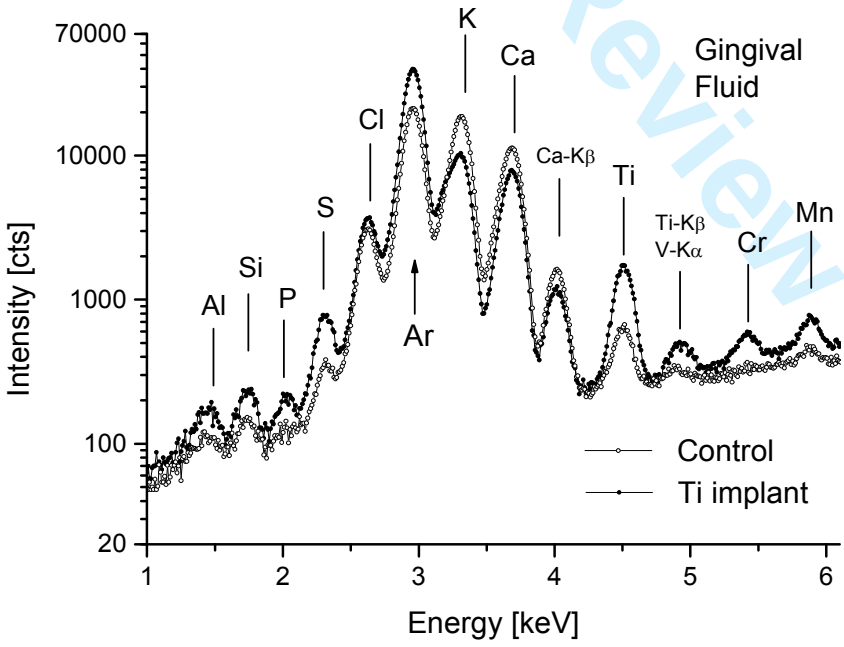

Figure 2b. Typical x-ray fluorescence spectra of gingival fluid from control and implanted groups. Note the different intensity (more than one order of magnitude) of the Ti Kα lines.

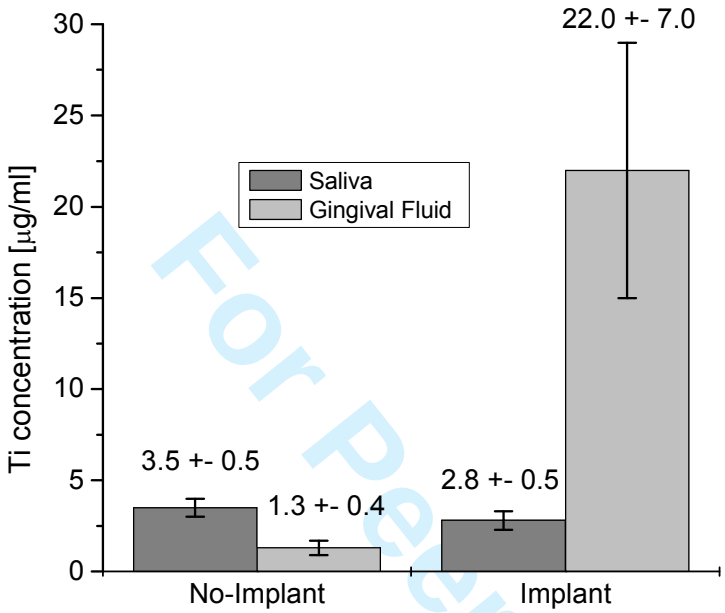

Figure 3. Bar graph of average titanium concentrations for saliva and gingival crevice fluid of the group of patients with dental implants and the control group.